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OUR MOULDY EARTH. A STUDY IN THE FUNGI
OF OUR ENVIRONMENT WITH EMPHASIS ON
WATER

William Bridge Cooke

Robert A. Taft Water Research Center
Cincinnati, Ohio

1970

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WITH EMPHASIS ON WATER

WM. BRIDGE COOKE

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ROBERT A. TAFT WATER RESEARCH CENTER
ADVANCED WASTE TREATMENT RESEARCH LABORATORY
CINCINNATI, OHIO

OUR MOULDY EARTH
A Study in the Fungi of Our Environment
with Emphasis on Water

Wm. Bridge Cooke
and
Collaborators

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U. S. DEPARTMENT OF THE INTERIOR
Federal Water Pollution Control Administration
Advanced Waste Treatment Research Laboratory
Cincinnati, Ohio 45226

ABSTRACT

Starting in early 1954, a total of 39 papers dealing with various aspects of the biology of the fungi in our environment have been published. These papers have appeared in a variety of journals, some of which are not readily available. To make these reports more readily available they have been assembled in this volume. An attempt has been made to present the work in an orderly fashion. We have started with a literature survey covering work published from other laboratories in North America, Europe, and Japan. This is followed by a survey of the techniques used in developing concepts about populations of fungi in sewage, polluted waters, sewage treatment systems, soils, and other aspects of the environment. The study of aquatic fungi in relation to polluted water is touched on briefly. Geofungi found in streams are considered in more detail, first considering seven streams not carrying other wastes, then three stream systems in the Ohio River Basin which carry acid mine-drainage.

In considering the occurrence of fungi in sewage treatment systems, the trickling filter is emphasized more than other treatment systems, but the results of sampling in several small activated sludge plants are given, as are the results of sampling in a waste stabilization pond system. It is also shown that fungi form part of the population of sludge digestion. The occurrence of fungi in paper mill wastes and the effects of such wastes on soil fungal populations are discussed. Those species of fungi known to produce disease in man and in plants are listed and discussed. The use of fungi as indicator organisms and a system of valency for fungi are described.

Carbon/nitrogen relationships, nutrient requirements, cellulose decomposition, BOD satisfaction, removal of ABS, yeast growth at 37°C, and growth and metabolism of fungi in an atmosphere of nitrogen are among the activities of fungi considered of importance.

Soils, especially samples from areas of minimum disturbance from man, have been analyzed, as have such varied environmental materials as used bedding, dairy products and a variety of other types of materials with which man comes into regular contact. Air near the influent box of a sewage treatment plant has also been surveyed for mold spore content. The final chapter considers the role of fungi in environmental sanitation. The reference lists of the papers assembled in this volume have been combined into one bibliography which completes the book.

FOREWORD

In its assigned function as the Nation's principal natural resource agency, the United States Department of the Interior bears a special obligation to ensure that our expendable resources are conserved, that renewable resources are managed to produce optimum yields, and that all resources contribute their full measure to the progress, prosperity, and security of America -- now and in the future.

This series of reports has been established to present the results of intramural and contract research studies carried out under the guidance of the technical staff of the FWPCA Robert A. Taft Water Research Center for the purpose of developing new or improved wastewater treatment methods. Included is work conducted under cooperative and contractual agreements with Federal, state, and local agencies, research institutions, and industrial organizations. The reports are published essentially as submitted by the investigators. The ideas and conclusions presented are, therefore, those of the investigators and not necessarily those of the FWPCA.

Reports in this series will be distributed as supplies permit. Requests should be sent to the Office of Information, Ohio Basin Region, Federal Water Pollution Control Administration, 4676 Columbia Parkway, Cincinnati, Ohio 45226.

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I am especially grateful to the publishers of the several journals and books from which these articles have been borrowed for permission to reprint the articles in full or with slight modifications. Also those who coauthored papers with me are thanked for granting permission for the reprinting of the articles. As time and assistance have been available, Mrs. Alice Proulx has kindly seen that the typing of the final manuscript has been carried out with a minimum of difficulty and error.

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OUR MOULDY EARTH

A Study in the Fungi of our Environment with Emphasis on Water

Wm. Bridge Cooke and Collaborators
Introduction

The fungi are found wherever there is organic matter to serve as a source of food supply. They are omnivorous and ubiquitous as a group, and some species know no limits of geography, or food supplies.

There are two principal reservoirs of fungus populations, the water and the soil. We think of the most primitive organisms as those which started in water and as they advanced in morphology they became capable of living on dry land, in this case, in the soil. Some fungi appear to have returned to the water from the soil, probably in response to a more convenient source of foods.

We can briefly review the principal groups of fungi based on broad habitat lines. The species known to be confined to the water in North America are listed and described by Sparrow (1960). A few of these are known to have migrated into the soil where they form a specialized and rather small segment of fungal populations. One of the more important of these land groups of aquatic fungi is the genus Pythium which Middleton (1943) has monographed. The fungi of this aquatic group are known to prefer clean water with high levels of dissolved oxygen. However, as will be noted later, a few species have been found to be associated with polluted waters.

As the fungi increased in complexity of morphology and of nutritional requirements, they came onto the land and their dependence

on water was reduced to high requirements of relative humidity for spore germination and growth, and high requirements for water for growth and development just as other organisms. Those which did not go on to more specialized habitats remained in the soil where they subsist on such varied types of organic matter as leaf litter, dead herbage, dead parts of animals, fecal matter, and similar substances. These are generally referred to as "soil" fungi or "geofungi". Many treatises have been written on these fungi or on the general habitat in which these fungi are part of a larger population. The biology of soil fungi is best summed up by a symposium edited by Baker and Snyder (1965). Many of the species are summarized by Gilman (1957).

Once on land, the fungi diversified their habitat requirements and they are now found in a great variety of places. One group of species from all classes is found on dung and fecal matter of herbivorous animals in particular but also of carnivorous animals. Cain (1934) and his students have summarized some groups and the studies are continuing. Special features of their biology can be found summarized by Ingold (1953, 1965), as well as in most text books of mycology. Benjamin (1959) has studied a special group of dung-inhabiting species and others are continuing this activity. The fungi in this group apparently fruit soon after the dung has been dropped or after it has been wet by rain. They have not yet been found in water, polluted or not.

Yeasts may have developed after the fungi arrived on land but because of various conditions some are returning to the water. Their life story has been told by Phaff, Miller and Mrak (1966).

As man's activities have increased, the fungi of the soil and other land habitats have adapted themselves to man's varied and increasing activities. Man is not immune to disease and as the fungi were taken into man's system through breathing the air, or as they were attached to man's body by the unclean practices of primitive men, they became adapted in part to man's body as a source of food. Emmons, Binford and Utz (1963) have summarized our knowledge of medical mycology excellently showing where most fungi which attack man have come from the soil.

Wang (1965) has found fungi abundantly in the slimes of paper mills where they are associated with various stages in the manufacture of paper. Malone and Muskett (1964) have found fungi to be pests of the seedsman both in the storage, germination tests, and planting of various types of seeds. Siu (1951) has shown the importance of fungi in the decomposition of cotton and other cellulosic fabrics. Wogan (1965) has indicated that some of the fungi are implicated in the production of toxic substances in foods. Prescott and Dunn (1959) have shown how fungi can be put to work in the making of a better world in which to live.

One of the more interesting types of adaptations of fungi in the soil involves the production of structures with which nematodes and rotifers can be trapped and eaten by so-called predacious fungi. Duddington (1957) has shown how the mechanisms work and how some species may be put to work to combat soil-borne pests of this type.

As more and more habitats become available to the fungi, more and more spores become available for dispersal. One of the commoner

means of dispersal for soil fungi which have also found habitats on dead plant parts, living plant materials, and other locations above the soil surface, is the air. Gregory (1961) has shown how, once a fungus spore has reached the air, it is transported and how it settles out of the air.

Many more habitats than those listed here have been taken over by the fungi. Their enumeration would be too lengthy to be included here. If now we return to the water we will find that Edmondson (1959) has enlisted the help of Sparrow in keying out the genera of aquatic fungi, and near the end of this Chapter 4, W. W. Scott has added a key to the genera of aquatic Hyphomycetes. These are presumed to be soil fungi which have returned to the water in which they now aid in the decomposition of fallen leaves and produce their spores at the surface of shallow pools. Notes on their biology will be found in chapters in the books by Ingold (1953, 1965). Ranzoni (1953) has reported on this group of fungi in California.

In addition to these more specialized fungi of aquatic habitats, many geofungi have returned to the water in response, it is presumed, to availability of nutrient supplies. It is these fungi which will form the basis for most of the following report.

Throughout the following report, mention of products and manufacturers is for identification only and does not imply endorsement by the Federal Water Pollution Control Administration or the U. S. Department of the Interior.

I. Literature on Fungi in Sewage and Polluted Waters

A. American and European Literature *

In developing a program for the study of the fungi associated with polluted water, sewage and sewage treatment processes, it was necessary to review (Cooke, 1954b) the work of earlier students of the biology of such habitats. Sphaerotilus and other bacteria are included because in the past they have been considered "sewage fungi" and they are referred to in this way.

In the text books dealing with the microbiology of water, such as Ward and Whipple (1918), Lackey and Phelps (1944), and recent bacteriology texts in which pollution is treated, fungi are either ignored or given only brief passing mention as organisms which may be part of the normal microbiota of such habitats. It should be noted that McKinney (1962) has devoted several pages to the fungi which he considers to be present but of relatively little importance in sewage treatment processes. Whipple (1927) indicated what groups of fungi could be found, gave the ecological classification of certain reported species of filamentous bacteria and molds according to the Kolkwitz and Marsson system, and illustrated several of the more common species.

Kolkwitz (1901, 1903) presented the first work done on the biology of Leptomitius lacteus, especially in relation to its habitat requirements in polluted water, and its cultural characteristics.

Shortly after installation at Waterbury, Connecticut of several trickling filters 7 ft. in diameter and 5 ft. deep in which stone 1-3/4

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to 2-1/4 in. in diameter was used, growth appeared which caused ponding. These were studied by Rettger (1906). The growths on the filter were red to black in color, although when washed they became white to reddish. They were obviously molds and when streaked on gelatin, after washing away most of the bacteria, mold colonies developed. From the description and drawings the mold was undoubtedly Geotrichum candidum. Rather than being caused by iron, as the author supposed, the red color may have been due to the presence of Fusarium aquaeductuum which did not appear or were not recognized in the plating growth. The growth was controlled by resting the filter, turning the stone, and hosing, or simply by resting the filters for a day or two, after which the dried flakes of growth washed off when the sprays were turned on.

Wilson and Calvert (1913) suggested that growths in streams can be used as indicators of pollution. Specific examples are given, such as the discharge of spent gas liquor, which caused Botrytis vulgaris to produce growths which blocked the channel of a small stream, and which in other places aided the growth of masses of Sphaerotilus natans, Beggiatoa alba, and Oospora or Fusarium solani. Bleach-croft refuse caused large developments of Sphaerotilus natans, and brewery effluents favored the growth of Monilia variabilis.

Weston and Turner (1917) found in the Coweaset stream, a tributary of the Taunton River which received the effluent of the sewage treatment plant of Brockton, Massachusetts, a large amount of Leptomit which

supported various types of life and which was supported by other types. It was found that certain organisms present in the stream assisted in maintaining an environment favorable to the development of Leptomit. When warmer temperatures occurred these organisms were inhibited and others became more abundant. The latter organisms appeared to produce conditions in the stream inhibitory to Leptomit. Leptomit developed profusely below the effluent drains, but never in the stream into which the Coweaset drained.

Tiegs (1919) presented brief notes on the biology of Leptomit, Sphaerotilus natans, and a new species of Penicillium, P. fluitans, isolated from industrial wastes. This species of Penicillium has recently been evaluated by Raper and Thom (1949) as a member of the P. frequentans series characterized by its ability to exist in solutions of 0. 25 N nitric acid.

Previous scattered literature reports of fungi in sewage plants were summarized by Cox (1921). He reported that Dunbar (1908) indicated that Peziza omphalodes and Pilobolus oedipus were present on filters, as well as in upper scums of septic tanks. In practically all cases the growths were considered of no value to good treatment and several types of removal techniques were recommended, such as flushing, resting the filters, or chemical control. In all cases observed, growth occurred in fall, winter or spring months. Cox studied the organisms on the filters at the Back River plant, Baltimore, Maryland. Among the organisms found were two unidentified fungi and the usual Zooglea, Sphaerotilus (Crenothrix), and Beggiatoa. From his descriptions the pink

fungus was probably Fusarium aquaeductuum and the grey mold Geotrichum candidum. Cox's work is largely only of historic interest.

Haensler, Moore and Gaines (1923) studied a vertical spray-type trickling filter plant at Plainfield, New Jersey, and found several fungi, of which five were chosen for special study. They studied materials from the surface and from 1-, 3-, and 5-foot depths in the filters. Material found was classified in five categories according to the texture of the mat. Subsurface and surface findings were tabulated according to the period of time in which collections were made. Penicillium sp. was more common in late summer, fall and winter at 1 ft.; Oidium sp. appeared more commonly in the first foot in the winter; unidentified fungus No. 3 (similar to the Sepedonium of the English workers) was a mid-spring fungus of the upper foot; Pythium sp. No. 4 appeared in the third foot more commonly, especially in the late winter and early spring; the fungus called Dictyuchus No. 5 occurred in winter and spring about equally abundantly at all depths. It was concluded that fungi were important in building up and binding the surface film of the filters.

Rudolfs and Trajkovich (1924) continued studies on the distribution of fungi in filter beds. They found that the fungi they studied increased in abundance as the temperature of the Imhoff tank effluent decreased during the year.

The same workers studied a species of Penicillium found on the filters. The culture was aerobic, used organic rather than inorganic nitrogen, did not produce ammonia, and was unable to decompose cellulose or to liquefy gelatin.

Starting as a study on the food of the insect Achorutes viaticus, the fungi of slimes of trickling filters and of a public-house cellar were studied by Bell (1926) in England. Slimes were placed in a 1:100,000 solution of formaldehyde for 96 hours and molds isolated in pure culture from them. In the Barnsley filter slime were found Torula rosea, Mucor mucedo, Oidium lactis, and Penicillium sp., whereas in the public-house cellar at Ripon, Yorkshire, were found Torula rosea, Mucor rouxii, Oidium lactis and Pencillium glaucum. On the basis of a demonstration that the insect fed on these molds, it was sent to nearly 40 sewage installations on three continents as a means of keeping the mold population under control.

One of the earlier reports of sewer line trouble resulting from mold growth in North America was that of Hall (1927), who reported that Sphaerotilus natans and Leptomitius lacteus caused serious trouble within a block or two of a creamery in Choteau, Montana. The trouble was apparently cleared up by the use of copper sulfate and continued periodic scrubbing to break up the film of growth.

In a primary treatment plant in Atlantic City, New Jersey, solids were settled out of sewage in tanks and pumped to drying beds. As soon as the sludge could be handled easily it was removed to vacant lots or under sheds. After further drying it was stored in darkened buildings where molds were encouraged to grow. It was not indicated which molds were present or whether the material with its microbial populations, which must have included bacteria, was sterilized before its sale as "fertilizer". Wigley (1930) indicated that this process was patented.

Prior to 1932 several species of filamentous bacteria and fungi had been reported in the taxonomic literature as having been obtained from polluted waters and sewage systems as well as, in some cases, clean water mains. Information on these was brought together by Butcher (1932) who, working in England, was especially interested in polysaprobic organisms. Under the term "sewage fungus" he grouped several species of filamentous and zoogloal bacteria, fungi, and stalked, ciliated protozoans. These were well-known components of the microbiota of polluted habitats. The species of fungi he treated deserve special consideration here because they are mentioned most prominently throughout the literature of pollution organisms whenever filamentous bacteria and fungi are discussed.

Filamentous Bacteria

Sphaerotilus natans Klützing, and its varieties listed below, according to Butcher occurred where growth was very rapid. S. natans var. natans, itself, is characterized by the presence of a gelatinous sheath around each filament. S. natans var. vulgaris Butcher has filaments which are found in a common gelatinous matrix and occurs as plumose or tassel-like growths 1 to 10 cm. long in free-running water. S. natans var. prolifera Butcher resembles Zopf's idea of Crenothrix polyspora. It occurs with the preceding forms, to the second of which it is similar. S. natans var. cladothrix Butcher (Cladothrix dichotoma Cohn) is characterized by the type of false branching of the filaments which has led many workers to include it in a separate genus. It occurs in habitats similar to those of the preceding varieties. S. natans var. depauperatus Butcher appears to be dwarfed form in response to a diminishing supply of nutrients characteristic

of the cleaner portions of streams down-stream from sources of pollution. S. natans var. transiens Butcher seems to be another form resulting from suboptimal environmental conditions, producing a growth form intermediate between Sphaerotilus natans and Zoogloea ramigera.

Zoogloea ramigera Itzigsohn forms spherical, branched, or lobed colonies and is well-known to sewage treatment plant operators. Whether or not it is a species which can be retained in good standing, or merely one stage in the life cycle of several species, is still an unsettled question, according to Butcher.

Beggiatoa alba (Vauch.) Trevis is a bacterium commonly found in sulfur-rich habitats, but which also occurs in the sewage-fungus community where the substratum is rich in organic matter.

Fungi

Leptomitium lacteus Agardh is a water mold with white or colorless hyphae without septa but with constrictions and branches. It forms tufts similar to those of Sphaerotilus natans, but is not slimy.

Fusarium aqueductuum (Radl. and Rabh.) Sacc. is reported as being rather rare, occurring in more acid waters. It has a septate, branched mycelium which produces typical crescent-shaped spores.

Protozoa

Carchesium spectabile Ehrenb. and C. lachmanni Clap. and Lach. are stalked, branched, colonial Vorticella-like ciliates which form colonies similar to those of Sphaerotilus and are thus included in the sewage-fungus community.

Butcher reports that pure culture studies have shown that all these organisms show a preference for nitrogenous compounds either in simple form

or as more complex amino acids or proteins. Where the more complex materials have been removed from the stream by degradation, growth becomes less obvious, but when carbohydrates are added to the stream, as in the case of sugar beet mill wastes, growth becomes more abundant. Growth is more dependent on nutrients than on temperature, although the blanket of sewage-fungus growth will extend farther downstream in winter than in summer. Sphaerotilus is more prevalent in England, whereas Leptomit appears to a greater extent in continental Europe, a fact which may be explained by the greater dilution of organic wastes in the larger streams of the continent rather than by the state of decomposition of this material.

Working with the bulking of activated sludge, Smit (1934) found four organisms to be associated with the process. Sphaerotilus natans appeared to be most important, although its identification was unsatisfactory, while Cladothrix dichotoma and a Leptomit-like organism seemed to be involved and "Geotrichoides paludosus" was associated with the sludge.

Under the chapter heading "Sewage Fungus," Butcher, et al. (1937) described the local and seasonal distribution of Sphaerotilus natans in the Tees and Skerne Rivers of northern England. To get a quantitative idea of this growth, weights of growth on glass slides exposed for 14 days throughout a year's period were recorded. The bacteria Beggiatoa and Zoogloea were recorded, but no true fungi were listed.

Tiegs (1939) brought together the pollution fungi of Germany under the general designation of "Abwasserpilze." The group was similar to the "sewage-fungus" community of Butcher and was treated from the standpoint

of required nutrients. By way of introduction it was stated that in the mid-nineteenth century even the layman had little difficulty in observing such growths, as indicated by works of the poet Wilhelm Raabe. The bacteria considered by Tiegs included Sphaerotilus natans, Zoogloea ramigera, Beggiatoa alba, and Thiothrix nives, while the fungi included Fusarium aquaeductuum, Leptomitius lacteus, and Mucor racemosus. Beggiatoa and Thiothrix are aerobic sulfur bacteria and were found in such habitats as municipal sewage plants, places where there is a predominance of sulfur, and in industrial wastes from plants in which sulfur is an important part of the process. Sphaerotilus did not occur to any extent in such places. These three groups prefer warmer temperatures than other bacteria in these habitats. Sphaerotilus prefers cooler temperatures, simple carbohydrates, and more complex nitrogen compounds, such as amino acids or their degradation products (ammonia or nitrates). It is less sensitive in nature to pH changes than in pure culture.

Leptomitius is the most important fungus in polluted habitats. It prefers nitrogen in proteinaceous form and occurs in either natural or artificially polluted waters. It appears to prefer streams in which the waste from slaughterhouses, breweries, or sugar factories is emptied. These streams often carry up to 200 p.p.m. organic matter. It prefers a cool temperature and a lower pH. It appears that Sphaerotilus is less specialized than Leptomitius as to its nutrient requirements.

Fusarium aquaeductuum is found in streams below the point of introduction of weakly acid materials. It contains carotinoid pigments, grows in light, and forms a red color on the substratum. The widely spread Mucor racemosus is common in sewage effluents rich in organic matter. It preferred

colder temperatures, a slightly alkaline pH, and was usually found only when using isolation technique.

Tiegs suggested that rather than treating sewage and industrial wastes with chemicals to prevent the growth of these organisms, the best method was to use settling basins or irrigated sewage fields. In further studies of these organisms it was not only necessary to know their nutrient requirements, but also something more of their environmental conditions.

Büchli and Huisman (1939), working with the problem of cleaning up the Limmat River, which received the sewage from the city of Zurich, Switzerland, tried three approaches after mechanically settling the sewage. Before treatment of the sewage was started the river bed and banks were coated with a thick blanket of white or gray material resulting from the growth of Sphaerotilus natans, Leptomitia lacteus, and Mucor. When an 80-m. long test channel was used several zones of growth developed in it, representing those organisms which could survive in the conditions of the material as it passed by. Zones of mold growth near the influent were followed by zones of several types of algal growth. Conditions of the habitat were tabulated and the various species of bacteria, fungi, and algae present were listed. To increase the effectivity of the trough in clearing the mechanically settled sewage, high-rate filtration provided partial purification and an activated sludge plant provided full biologic purification.

Lackey and Wattle (1940), in a recent treatment of the biology of Sphaerotilus natans, summarized previous work and presented studies on this organism in relation to bulking of activated sludge. They found that five different species of Sphaerotilus, possibly only physiological variants

of one species, had been associated by various workers with pollution of one sort or another and with sludges in sewage treatment plants and experiments. The activity of this species as a bulking sludge producer in the laboratory was greatest in the presence of excesses of mono- and di-saccharides when an available nitrogen source was present. Using a large number of nutritional variants in activated sludge, sterile raw sewage, medium "L", and synthetic sewage, an attempt was made to determine whether or not additional nutrients were present in sewage which would produce such large growths. No substances other than mono- or di-saccharides produced heavy growth of the organism. An attempt was made to find a substance to control the growth of Sphaerotilus, especially when it was responsible for bulking of sludge, and not inhibit other members of the sewage-fungus community. It was found that the dye malachite green was inhibitory to Sphaerotilus. If this was too expensive for general use some form of chlorination of returned sludge would be best for most treatment plants.

Schade (1940) and Schade and Thiman (1940) presented thorough studies of nutrition and metabolism of Leptomitus lacteus. Using modern physiologic and biochemical techniques, their work has brought up to date the work of Kolkwitz and Trommsdorf on this organism and gives the reader an insight into some of the reasons why this organism prefers the more polluted habitats in which it is found.

In a brief text on sewage treatment, Rudolfs (1941) summarized the work done on molds in sewage at Rutgers University.

Tomlinson (1946) found that the stone of trickling filters was covered with a film of organisms including algae, fungi, and bacteria. This film

could, under certain circumstances, form a blanket between stones. The fungi in the Minworth, England, filters included species in the genera Fusarium, Phoma, Sepedonium, and Oospora. The film varied in composition and thickness from one observation period to another. Fusarium formed tightly adhering mats on the stones and Phoma occurred with it, whereas Sepedonium and Oospora formed continuous mats which caused ponding when mats were of large size. A pair of filters was operated in series experimentally. A 3-to 4-day alternation of filters in the series was used. A series of glass slides was exposed on the surface of these filters and periodically weighed to determine the effect of alternation on the material growing on the filters. A set of slides exposed on a single filter not receiving effluent from another filter showed a gradual increase of film on the slides for several months, then a leveling off, accompanied by fluctuations due to causes found in single-filter operation. The slides on the pairs of filters in series, on the other hand, showed an additional periodic fluctuation as the filter received first the settled sewage, then the settled effluent from the other filter.

Sphaerotilus natans was shown by Ruchhoft and Kachmar (1941) to be an obligated aerobe with considerable variability in viability and metabolism. Experimentally, bulking was shown to be a response of sludge organisms to a sudden disturbance in the biological equilibrium, especially as to three primary factors including sludge, nutrients, and rate of oxygen supply. It was found that in certain cases of bulking the disturbance producing it was accompanied by a great increase in Sphaerotilus, although other organisms present in activated sludge could cause bulking under other disturbed conditions.

Weston (1941) discussed the role of aquatic fungi in hydrobiology. He dealt almost exclusively with those fungi which occur in non-polluted water or water polluted only with dead or discarded remains of tissues of plants growing in the immediate environment of the streams, ponds, or lakes under study. In such places many kinds of fungi live as saprobic invaders of dead tissues, or parasites on plant or animal plankton species, or predators on planktonic species.

Rudolfs and Heukelekian (1941) discussed briefly the microbiology of sewage and sewage treatment plants. They mentioned the role of various types of microorganisms in hydrogen sulfide production, sludge digestion, slow sand filtration, trickling filters, and the activated sludge process. Fungi were mentioned as playing a part in the film formation on the stone of trickling filters.

Reynoldson (1942) found that in a double filtration plant at Huddersfield, England, there was a correlation between growth of the mold Oospora and the fly Psychoda, which followed a temperature pattern. In the primary filter there was heavy fungal growth with ponding and sludge accumulation, but with a lowered fly production. In the coarse secondary filter there was no ponding, the thickness of the fungus blanket was greatest in winter and spring, and the flies followed a normal pattern with temperature, except for a late summer period when they were reduced, apparently by lack of food.

Heukelekian (1942) used baker's yeast in seeding open and closed vessels containing ripe sludge to determine the effect of yeast on stimulating digestion and stabilization of sludge. Tests were run at 20°C. and daily observations were made. When 2 g. of yeast per liter of sewage were added

in open vessels, the 5-day B.O.D. increased slightly, although it decreased by one-third in uninoculated controls. In a second test, using closed vessels, 0.1 g. of yeast per liter of sewage was used and at the end of 48 hr. the 5-day B.O.D. was slightly higher in inoculated vessels than in uninoculated controls. It was concluded on the basis of 5-day B.O.D. measurements, reduction of volatile matter, and gas production, that yeast does not aid in the stabilization of sewage kept under either aerobic or anaerobic conditions, and that its addition to a new digester would have little, if any, value.

Wilson (1943) presented data to indicate the relative importance of the binding organisms, Beggiatoa alba, and fungi as a group, and various free-living and scouring organisms in the Glenwood City, Wis., and the Owatonna, Minn., trickling filters.

Lackey and Dixon (1943) analyzed the populations of 11 contact aerator plants of the Hays process type. Seventy-one distinct groups of organisms were recognized, but the fungi found were left unidentified. These were found usually in the manner of the plankton specialist studying samples in the Sedgwick-Rafter cell.

Tomlinson (1946) studied filters at Minworth in which the three components of the film were a zoogloal bacterium, Ascoidea rubescens, and Sepedonium sp. During the summer the film developed to twice the extent of that in the winter because of the greater amount of growth of Ascoidea and the bacterium. The Sepedonium developed in the winter. On the basis of total dry weight of the film in the top 2 ft. of the filters, a greater amount was found in the single than in the double filters.

Wilson (1949) described the conditions under which the microbiota of sewage

treatment plants live. In so doing he emphasized three terms: binding, free-living, and scouring, which applied to the habit of the various types of life in the filters. It can be noted that of these categories the fungi of filters fit into the first. In many filters fungi may be the most important binding organisms, whereas the filter fly larvae, as scouring organisms, remove the fungus mycelium and other parts of the film for food. In his analyses of the various habitats Wilson indicates that certain unidentified species of fungi existed.

Beardsley (1949) made an attempt to control slimes in the sewer system of Los Angeles, Calif., shown by Pomeroy and Bowlus (1946) to cause excess production of sulfides. The occurrence of slimes on walls of sewers was diverse and their effect was variable with respect to time and place. It was found that certain types of organisms were suppressed by chemical treatment while others were unaffected. Organisms responsible were thought to include Leptomitius lacteus and possible Apodachlya brachynema, as well as the bacteria Zoogloea ramigera and Beggiatoa alba. It was suggested that possibly certain Myxomycetes were also involved.

Harvey (1952) found 21 species of aquatic fungi associated with streams of the Little Miami River basin. Of these, one occurred frequently enough to be considered a possible inhabitant of the septic zone of Lytle Creek, the stream receiving the effluent of the Wilmington, Ohio, sewage treatment plant.

The reports of the (British) Water Pollution Research Board (1951, 1952, 1953) for 1950, 1951, and 1952 presented information concerning the growth and physiology of fungi of small trickling filters. Where the filter medium was only 3/4 in. to 1-1/2 in. in diameter, more ponding, due to fungi, occurred

than in the medium which was 1 1/2 in. to 3 in. in diameter. It was also shown that where the filters were operated at 800 and 1,000 g.p.d. per cubic yard only 13 to 14 per cent of the film was fungus mycelium, whereas in filters operated at 200 to 600 g.p.d. per cubic yard this material varied between 14.5 and 30 per cent.

Because of the importance of at least 10 species of fungi in producing ponding effects in the upper layers of small filters, experiments on nutrition of the more important species were undertaken. A species of each of Sepedonium, Oospora, Sporotrichum, and Fusarium aquaeductum was selected for special study in 1951. Basic media, pH ranges, mineral requirements, and vitamin nutrition were studied. It was found that Sepedonium could grow in a pH range of 4 to 9.5, with optimum of 7 to 8. Fusarium and Oospora required no outside source of vitamins, whereas Sporotrichum required thiamine, and Sepedonium required biotin in addition to thiamine. It was found that these vitamins and certain trace elements were present in sewage. Sepedonium required oxygen, since it grew well in aerated culture, poorly in quiescent liquid cultures, and hardly at all when nitrogen gas containing 1.2 per cent oxygen by volume was passed over the surface of the medium. In an artificial filter using glass balls and an artificial sewage-like nutrient medium, the filter was found to be effective only after large growths of the fungus had developed after at least 15 days.

In a glucose-casein hydrolysate-mineral salt medium it was found, in 1952 studies, that Fusarium aquaeductum and Sporotrichum had a pH range of 3 to 10, with optimum growth at pH 4 to 9. Oospora fragrans had the same range, with optimum at pH 3 and no growth at pH 2; Sepedonium had

the narrow optimum range of pH 7 to 8. The weight of Sepedonium after 10 days was found to be directly related to oxygen supply over the range of 2 to 15 per cent of the gas mixture. Nitrogen requirement studies indicated that although Sepedonium preferred complex organic nitrogen compounds, Sepedonium and Ascoidea rubescens required organic nitrogen. Oospora fragrans and Sporotrichum could use ammonium salts in addition, and Fusarium aqueductum could also use nitrates. Further studies on minor elements indicated a need for, or tolerance of, 11 elements. Zinc and calcium by themselves were toxic to Sepedonium, while in combination with the other 9 elements used, they gave increased yields. Using the optimum mineral salts solution, results of the vitamin experiments reported previously were confirmed. Using settled sewage, Sepedonium gave better growth than the use of ashed sewage, which may be explained either as the better availability of growth-promoting substances or of trace elements.

Hesseltine (1953) surveyed the surface stone of a trickling filter handling the industrial waste from a chemical laboratory and an antibiotic plant at Pearl River, N. Y. He found three fungi of importance and described them morphologically. These included Geotrichum candidum, Fusarium aqueductum, and a representative of a new genus, Subbaromyces splendens.

The more important species reported by various workers in the period 1901-1953 are summarized in Table 1. The liberty has been taken of identifying species where these are recognizably described in the literature but treated as unknowns. Certain names are brought into conformity with recent ideas of nomenclature. Although the list is incomplete, it indicates that the species of filamentous bacteria and fungi associated with polluted

Table 1. Important Fungi Associated with Sewage and Polluted Waters.

Location	Observer	Year	<u>Leptomitius</u> <u>lacteus</u>	<u>Geotrichum</u> <u>candidum</u>	<u>Sepedonium</u> <u>sp</u>	<u>Fusarium</u> <u>aquaeduct-</u> <u>tum</u>	<u>Penicillium</u> <u>app</u>	<u>Sphaerotilus</u> <u>natans</u>	<u>Bezzia</u> <u>alba</u>	<u>Zoog-</u> <u>loea</u> <u>real-</u> <u>gira</u>
Germany	Kolkwitz	1901	x	-	-	-	-	-	-	-
Connecticut	Rettger	1906	-	x	-	x	-	-	-	-
Germany	Teigs	1919	x	-	-	-	x	-	-	-
Massachusetts	Weston	1917	x	-	-	-	-	x	-	-
Maryland	Cox	1921	-	x	-	-	-	-	-	-
New Jersey	Haensler	1927	-	x	x	x	x	-	x	-
England	Bell	1926	-	x	-	-	x	-	-	-
Montana	Hall	1927	-	-	-	-	x	-	-	-
England	Butcher	1932	x	-	-	-	-	-	-	-
Germany	Tiegs	1939	x	-	-	x	-	-	x	x
Switzerland	Blochliger	1939	x	-	-	-	-	-	x	x
England	Tomlinson	1941-6	-	-	-	x	-	-	-	-
England	Research Board	1951	-	x	x	x	-	-	-	-
New York	Hesselstine	1953	-	x	-	x	-	-	-	-

water and sewage are very wide-spread.

From this literature survey concerning fungi of pollution habitats it appears that the workers who have made these studies have done so with materials which have appeared obvious to the field collector. No attempts, or only very cursory attempts, have been made to find those fungi not so easily seen. Fungus mycelia are very similar from species to species either in the group of Phycomycetes, where septation occurs rarely, or in the Ascomycetes and Fungi Imperfecti, where the mycelia are commonly septate or "segmented." Species cannot be separated on this character and must be cultivated to the extent that they will produce reproductive structures and spores by means of which they can be identified. In doing this, different media and techniques are required and several species may become evident where only one appeared before. In preliminary samplings at several sewage treatment plants of two types it has been found that more than 50 species of fungi are associated with such habitats, either as permanent residents or as casual transients. More than 100 species of molds have been found in a stream subject to pollution from the effluent of a sewage treatment plant of the primary type. The problem of the activity of fungi in polluted habitats is not that of discovering the activity of one or of five molds, but of determining the activity of a great many molds, each of which has a different set of requirements, but many of which are more or less physiologically related.

Supplement

The following reports have come to my attention since completing the preceding literature review.

Holtje (1943) briefly summarizes the biological components of slimes found on sewage sprinkling filters in a New Jersey treatment plant. Fusarium and Leptomit are mentioned and illustrated. He was of the impression that fungi and bacteria are in a continual struggle for dominance with the fungi predominant in the winter and their film sloughing off in the spring.

Kolkwitz (1950) published an expanded version of his "Ecology of Saprobes" in which he outlines his classification of organisms of waters whether these were polluted or not. Species listed under the various saprobic categories are accompanied by discussions of their ecology.

Wurtz (1957), in a report on fungi, bacteria and algae in polluted waters in France, discussed Leptomit lacteus at length, and gave additional information on the distribution of Saprolegnia, Mucor, Fusarium, other molds and yeasts in the streams of that country. A key is presented for the identification to the ordinal and generic levels of these fungi.

In Sweden, Stjerna-Pooth (1957) found that Achlya prolifera was important in sewage polluted streams. The occurrence and ecology of this fungus are discussed at length.

Coudert and Saez (1958) at Lyon, France, found several kinds of fungi in public fountains, medical laboratories, apartments, and in industrial wastes. Species found are listed and the habitats discussed briefly.

Branco (1962) comments briefly on the fungi which can be found in sewage and sewage polluted waters. Her note is largely a review of material published in North America.

Hynes (1960) recognizes the presence of fungi in polluted stream

water but to him these are of little importance, apparently, unless they bloom out into more or less massive growths indicating a large amount of organic pollution entering the stream in the vicinity.

This review of the literature can be concluded with a brief mention of McKinney's (1962) textbook "Microbiology for Sanitary Engineers". The author recognizes the occurrence of fungi in sewage, sewage treatment systems, and polluted waters and includes a chapter on these organisms. However, he gives them little credit for sewage purification since he considers them strict aerobes and since he attributes to the bacteria the greater role in the breakdown of organic wastes which can be found in sewage polluted waters.

Following the beginning of intensive sampling at Cincinnati in 1952, a number of reports have been published on the fungi which occur in sewage, polluted water, sewage treatment systems, undisturbed soils, soils and streams to which industrial wastes have been added, and other types of environmental habitats. The results of this work are summarized in this brochure following the presentation of a review of recent Japanese literature on fungi in water, both clean and polluted.

B. The Japanese Literature

Although discussed separately, these 42 papers are included as a unit because they form the basis for a study of water quality unlike any other previously found. From 1956 to date, the student of mycology and mycologist, Shizuo Suzuki, and his colleagues, have worked extensively on clean and polluted waters in the vicinity of Tokyo, Japan, studying the distribution of bacteria and fungi with relation to different types of clean water lakes, and different types of pollutants added to river waters. Work with clean waters from areas of volcanic activity is largely related to studies of aquatic Phycomycetes, aquatic Hyphomycetes, other filamentous fungi, and bacteria, found in individual or groups of lakes. This includes clean waters carrying large amounts of minerals making them more or less comparable to our waters carrying acid mine drainage.

So far as I am aware, this is the first series of papers in which a quantitative method has been applied to the occurrence of zoospore-producing aquatic Phycomycetes in any type of water. The technique appears to be based on the assumption that each hypha arising in a colony of fungi developed on a hemp seed was produced from the colonization of that hemp seed by one zoospore. Therefore, the numbers of hyphae on the hemp seed represent the numbers of zoospores in the tested unit of water brought from the particular body of water to the laboratory, but not necessarily tested directly in the field beside or within the body of water. Obviously, the technique cannot be used if the bait is placed directly in the body of water. When waters from various depths

in lakes or rivers are tested, samples from several levels in the body of water are removed with the Eckman apparatus. In most cases chemical and physical analyses of the waters tested for microorganisms are reported. The populations of bacteria and fungi are used as supplemental characteristics in the description of the tested bodies of water.

Ten species of aquatic Phycomycetes were isolated from waters of Senshun-ike Pond, Tokyo (Suzuki, 1960h) in the period from July 1955 through June 1956. Some appeared only occasionally, some in the colder months, some in the warmer, and two were present throughout the year. Maximum yields of zoospores produced 911/10 cc in spring and autumn, minima yielded 32/10 cc, in summer and winter. Water temperature was thought to play an important role in zoospore production as well as the increase and decline of lacustrine bacterial populations.

Lake Nakamura (Suzuki, 1960i, 1961j) was sampled at monthly intervals from October 1957 to September 1958. It was found that 7 species of aquatic Phycomycetes were present, with a maximum of 65 zoospores per 10 cc of water in early winter, and a minimum of no zoospores per 10 cc of water in the summer. A homogeneous distribution of zoospores occurred during the lake's circulation period, a stratum type during the stagnation period of the lake. Seasonal changes in distribution differed with each species. However, 4 species were found mostly during the winter and 2, including the dominant Pythium sp., were seen throughout the year with their maximum in the winter. Zoospore production was affected by water temperature and by lacustrine bacteria. Optimum temperature for zoospore production varied with each species.

In Lake Shinseiko (Suzuki, 1960g) similar conditions were found except that there was an increase in numbers of zoospores in spring and autumn, with a decrease in summer and winter. Of the 7 species found most occurred from autumn to early spring, one in summer and one throughout the year with maxima in spring and autumn. During the stagnation period two were distributed in the surface layer, while during the circulation period one was found mostly in the bottom layer.

Following a survey of 68 lakes in Japan, Suzuki (1961c) was able to record three species of Achlya, of which two were rare, A. racemosa appearing only in the winter and A. americana only in the summer. A. flagellata was common, widely distributed, appeared throughout the year and in all types of lakes sampled.

In a group of inorganic acidotrophic lakes (1961a) with pH ranges from 1.9 - 5.8, with high quantities of minerals, 5 aquatic Phycomycetes were found. Zoospores to a number of 0-1800/10 cc of water were found in increasing numbers in increasing degrees of acidity. Fungi were rare or absent in bottom muds. The number of species increased with decreasing pH values.

On the slopes of the Volcano Bandai (Suzuki, 1960d, e; Suzuki and Nimura, 1960a, b, 1961c, d) are a number of groups of fresh water lakes. Six of these groups were studied from the points of view of mineral content, certain physico-chemical factors, and the fungal populations. Certain fungi were found to be restricted to lakes with varying degrees of acidity or mineral content. Most of the lakes fell into such categories as acidotrophic, ferotrophic (with large amounts of iron),

dystrophic and harmonic. Larger numbers of fungi were found in harmonic lakes whose reactions ranged around neutral. Some aquatic Phycomycetes and Hyphomycetes were found to be restricted to acidotrophic lakes, others could not survive in more acid conditions than those found in harmonic lakes.

Conditions similar to those found in the lakes on Bondai Volcano were found in the lakes of Volcano Nikko (Suzuki, 1961d), the Shiga Lake group (Suzuki and Hatakeyama, 1960), and the lakes of the Shiga Volcano (Suzuki and Nimura, 1961a).

Suzuki (1961g) studied the bacteria and fungi of Lake Katanuma in the Katanuma Volcano. The water of this lake has a pH of 1.9 because of the quantity of sulphuric acid present. Penicillium citrinum was the only fungus found. In the laboratory, spores of this fungus were found to survive at least 7 month's exposure to water of this acidity while other fungi, including members of the Mucorales, died within a few days.

It was found that the genus Pythium (Suzuki, 1961f) is widely distributed in Japanese lakes. A seasonal fluctuation is shown with a maximum production in winter, the minimum coming in summer. Distribution was related to lake type, the species being more common in harmonic lakes, rare in dystrophic and acidotrophic lakes.

The aquatic Phycomycetes genus Aphanomyces (Suzuki, 1961c) was found to be widely distributed in Japanese lakes. It occurs in bottom muds as well as in water. Seasonal fluctuation varies with each species. The distribution of the genus is also related to lake types: species were abundant in harmonic and dystrophic lakes, rare in acidotrophic lakes, species were very abundant in eutrophic lakes but rare in mesotrophic and oligotrophic lakes.

Aquatic Hyphomycetes (Suzuki and Nimura, 1961a) were found to follow a similar distribution pattern. While most species are confined to dystrophic and harmonic types of lakes, a few species grow in acidotrophic lakes. Those species which grow in harmonic lakes cannot sporulate in water of acidotrophic lakes.

The aquatic fungus Saprolegnia monoica var. acidamica has been found (Suzuki, 1961i) to occur in highly acid waters which also carry heavy loads of mineral ions. It is found only in acidotrophic lakes and seems to be more adapted to lower water temperatures than most similar fungi.

Although absent from two, it was found (Suzuki and Nimura, 1962) that six species of aquatic Hyphomycetes are found in inorganic acidotrophic lakes in Japan. The amounts of iron and pH values form the major restrictions on distribution of these fungi according to laboratory experiments.

Diurnal distribution of zoospores of aquatic fungi (Suzuki, 1961b) in a shallow lake were related to oxygen supplies and light. In spring and autumn, distribution was related to oxygen supplies, being concentrated in water layers rich in oxygen. On a clear day zoospores were most abundant in the bottom layer, on a cloudy day in the surface layer, and on a rainy day they were distributed homogeneously throughout the water of the lake.

The vertical distribution of fungus zoospores (Suzuki, 1961-l, Suzuki and Nimura, 1961e) was of two main types: homogeneous distribution occurred during the circulation period of the lake, stratum

distribution during the stagnation period. All species were found in the epilimnion during the stagnation period, while variations according to genus were found during the circulation period.

While the optimum temperature (Suzuki, 1963d) for zoospore production of five species of aquatic fungi collected in Japanese lakes lies between 16° and 20°C, the critical temperatures below or above which zoospores are produced differ for each species observed.

Studies made between August and November 1957 in the mesotrophic Lake Yamanakako showed (Suzuki and Hatakayama, 1961) that representatives of three genera of aquatic fungi are present. Maximum numbers of zoospores were present in mid-autumn, minimum numbers occurring in summer and late autumn samples. Seasonal fluctuation was related to species occurrence. Aphanomyces was more abundant in the summer, Saprolegnia in the autumn, while Pythium species were present regardless of season. While zoospores were present only in the epilimnion in the summer, in the autumn they were generally distributed at all depths tested. Bottom muds were rich in species in autumn but Pythium was preponderant regardless of season.

As a result of numerous surveys of lakes and streams in Japan (Nimura and Suzuki, 1962), a list of 21 species, including 1 variety, belonging in 16 genera, of aquatic Hyphomycetes has been developed.

In a stream polluted by municipal sewage wastes, aquatic fungi (Suzuki, 1960a) were found in both polluted and unpolluted reaches. Species of Saprolegnia and Achlya were found below the sewage outfall, Aphanomyces and Pythium were restricted to the cleaner reaches of the stream above the outfall.

In the Kiryugawa River receiving sewage from Kiryu City (Suzuki, 1960f), it was found that the rate of buildup of bacteria is higher in undiluted sewage than in that diluted by river water. Above the sewage outfall Aphanomyces was a common organism, while at the outfall Monilia sp. was a dominant fungus, and below the outfall a number of organisms occur. Among the bacteria, Beggiatoa sp. and Sphaerotilus natans, among the aquatic Phycomycetes, Leptomitius lacteus, and among the filamentous fungi, Monilia sp., are common organisms in the 50-150 M stretch of the river below the sewage outfall.

The stream serving Fujisawa City (Suzuki and Nimura, 1961b) and carrying its domestic wastes was studied. Largest number of bacteria occur downstream from the sewage outfall. Fungi are abundant at each sewage outfall point. Of the aquatic Phycomycetes, Aphanomyces sp. was common in clean water, while Pythium sp. was common in sewage-polluted water. Among the abundant microorganisms in sewage-polluted water were Beggiatoa sp. and Sphaerotilus natans among the bacteria and among the fungi, Mucor fragilis, Monilia sp., and Trichoderma koningi.

A study of the Arakawa River (Suzuki, 1960b) shows that in upper reaches aquatic Phycomycetes are rare but increase in number downstream especially in the vicinity of Kumagaya whose domestic and industrial wastes are dumped in the river. These organisms decrease downstream as urban and industrial pollution increases. The zoospores vary in quantity, the maximum occurring in winter, the minimum in summer. Seasonal fluctuation occurs according to species. Zoospores were distributed uniformly vertically but spores of Saprolegnia monoica were found mostly in surface water, of Leptomitius lacteus mostly on the bottom.

Fourteen wells in Tokyo and its suburbs were studied (Suzuki, 1961h). In these waters were found relatively high numbers of bacteria, aquatic Phycomycetes and filamentous fungi. Bacteria isolated from water from some of the wells were inoculated in waters of all the wells and showed a rate of increase at 30°C after 24 hours of 6-780. It is indicated that there are adaptable bacteria in each well. Pythium sp. and Saprolegnia sp. were dominant among the aquatic Phycomycetes; Mucor and Penicillium species among the filamentous fungi.

In waters of a river polluted by candy factory wastes (Suzuki, 1961e, Suzuki, et al., 1960) large numbers of fungi were found at the effluent point where the aquatic fungi Saprolegnia sp. and Pythium sp. are common and the filamentous fungi Monilia sp. and Mucor fragilis were abundant. In the river bottom 50-80 M below the outfall, Sphaerotilus natans is dominant with a few colonies of Monilia sp. and Mucor fragilis also appearing.

Studies were made in a river receiving paper mill wastes near Tokyo (Suzuki, 1961e, Suzuki and Suzuki, 1961). Larger numbers of fungi occur above the point of wastes outfall from the mill than at or below that point. There were no aquatic Phycomycetes associated with these wastes and the dominant, if not the only, organisms were Sphaerotilus natans and Trichoderma koningi. Physiological studies indicate that for fungi organic nitrogen is preferred in a medium based on Czapek's agar. The toxicity of the wastes from the mill was determined by the IC₅₀ method developed by Suzuki. This technique demonstrates the "inhibiting concentration for microorganisms", the concentration at which

half the microorganisms are inhibited (or killed?). In this case the IC_{50} was found to be $1/8$.

A study of the pollution on rivers entering Tokyo Bay has been carried out (Suzuki, 1962, 1963a, b, c). The principal interest in this study was on the occurrence and distribution of general bacteria, specialized bacteria, filamentous fungi and aquatic fungi. In general, in the cleaner upstream areas fewer individuals and species of organisms were found. As pollutants from paper mills, candy and other factories, and as municipal sewage entered the river, colony counts and numbers of species increased and these dropped off again at the mouths of the rivers where they entered Tokyo Bay and blended with salt water. More kinds and numbers of fungi were found in water on the surface of the streams than in shore waters and bottom muds although some species were restricted in activities to these locations. For growth, the fungi found require nitrogen-bearing compounds.

In summarizing (Suzuki and Suzuki, 1962) early work done on the occurrence of fungi in polluted water and sewage in Japan, it was noted that Leptomitus lacteus had been isolated 4 times from candy, paper mill wastes and sewage; Mucor fragilis 14 times from candy and alcohol plant wastes and sewage as well as from several locations in a sewage treatment plant; Geotrichum candidum 28 times from candy, alcohol and paper mill wastes, municipal sewage and sewage treatment plants; Trichoderma koningi 12 times from candy and paper mill wastes, sewage, and a sewage treatment plant; and Fusarium aquaeductuum 3 times from alcohol factory wastes and sewage.

Physiological studies were made (Suzuki, 1961k) on Monilia sp. Best conditions for growth of the fungus, of which 19 strains were isolated from sewage-polluted streams, industrial wastes, sewage and sea water, are described for temperature, carbon, and nitrogen sources.

Because of its prevalence in activated sludge, Geotrichum candidum was studied (Suzuki and Tatsuno, 1964) to determine something about its activity. The authors found that under the conditions of their experiments the ability of this fungus to break down organic matter was not as strong as under other experimental conditions. They thought that it does not play an important role in the activated sludge process.

Aluminum sulfate is used (Suzuki, Tatsuno and Ueno, 1964) as a coagulant for particulate matter in raw water entering certain industrial processes and in water treatment plants. In one situation it was found that aluminum sulfate slurries were infected with Trichoderma koningi, a common organism of clean and polluted streams. The addition of copper chloride or other disinfectants effective against this organism was recommended.

II. Techniques for Study of Fungi*

A. Collection of Samples

General

For determining the presence of a fungal biota in any habitat, grab samples are satisfactory. A rounded picture of such populations in a stream may be obtained by grab sampling the water, the sediment in pools and in riffles, being careful not to obtain gravel and stones, and in the always-wet bank soil, at points used for other types of sampling in the same or similar projects. Glass or plastic vials, soil cans, or other containers may be used. A volume of at least 45 milliliters of material is required. The container should be clean and the sample should be handled aseptically because of the possible addition of organisms not pertinent to the problem. For this reason try to avoid contact between water or soil in the sample and any part of the body, especially the fingers.

At the chosen sites, samples are obtained by scooping into the sample container the water, mud, sand, or soil to be examined. It is not possible to get bottom samples without water; however, try to get a minimum of water and a maximum of bottom mud or sand. For bottom sampling in water deeper than elbow depth, any available sampling device can be used. In bank-soil sampling excess water is not necessary, although it may come with the sample in times of heavy precipitation or flooding. The container is closed with its proper cover, sludge, dirt, etc., may be wiped off, it is then rinsed off, numbered and returned to the laboratory. The container should not be completely air tight.

* Materials used in Chapter II, sections A, B, C, and D, were taken from A Laboratory Guide to Fungi in polluted waters, sewage and sewage treatment systems. Public Health Service Publication 999-WP-1, 1963. This summary brings together techniques published in earlier papers, including Sewage and Industrial Wastes 26: 661-674, 1954; and Antibiotics and Chemotherapy 4: 657-662, 1954.

Aquatic Fungi

To demonstrate populations of aquatic fungi, parts of samples obtained as described above may be used, larger grab samples may be brought to the laboratory for the purpose, or "baits" may be exposed in the stream, pond or lake to be sampled.

Cylinders can be prepared made of quarter-inch mesh hardware cloth stoppered at each end with corks. From one of the corks a steel wire skewer holding a piece of beef, a piece of apple, a whole date and a rose hip or a hawthorne fruit, projects into the cylinder. The cylinder, floating partially submerged, is tied to some permanent object on the shore and left for a week. Sites are chosen where bridge pilings or living shore shrubs can be used as the objects to which to tie the cylinders. Non-decomposable string such as nylon fishing line can be used for tying. Care should be taken to guard against areas frequented by curiosity seekers who tend to destroy bait containers such as these. At the end of a week the baits are returned to the laboratory, preferably in a bottle of water obtained from the point at which the baits were exposed. The bottle should not be tightly sealed to allow an exchange of air.

The most popular baits used by mycologists dealing with aquatic fungi are hemp seeds. These can be exposed in natural bodies of water in one of two ways. Hohnk and Bock (1954) suggesting making small bags of loosely woven nylon cloth which are suspended in the body of water to be sampled. Autoclaved halved hemp seeds are included in these bags and should be colonized by zoospores of the aquatic fungi living in the

body of water. Cooke and Bartsch (1960) used aluminum tea balls which float. In the teaballs are placed autoclaved halved hemp seeds and the tea balls are tied to branches of shrubs along the shore of the body of water to be sampled. After 3-7 days exposure the hemp seeds may be returned to the laboratory for further processing. The tea balls are floated in a container of water from the sampled location and on return to the laboratory are placed with fresh hemp seeds in sterile lake or carbon water.

Geofungi

To sample fungal populations in materials from sewage treatment processes of any kind, the techniques for soils and stream samplings described above are used. Grab samples of sewages, waters, sludges, scums, bottom materials from settling basins, etc., can be obtained in any type containers that hold more than 50 milliliters of sample. Scraping 50 milliliters of slime from a tricking filter stone on an intermittent low rate filter is tedious; it is less tedious on a continuous high rate filter. For any one location, if periodic sampling is to be carried out, only one set of samples at the 50 milliliter or more level is required, provided that the subsequent samples in the series are taken from approximately similar materials.

Fifty milliliters or more of sample are required to obtain a definite basis for correlating the results of isolations from samples. For purposes of reporting, the populations should be listed as numbers of colonies per gram dry weight of sample (with the possible exception

of water samples). The number of colonies per milliliter of sample is also obtained, but because each type of sample contains varying amounts of dry matter, the gram of dry weight is a more realistic basis of comparison than the milliliter of liquid or moist sample.

For obtaining oven dry weights of the solids in each sample, 15 milliliters of the sample are used in preparing the dilution from which plates for fungal growth are to be poured. Of the remainder, 15 milliliters are placed in each of two soil cans which have been previously cleaned and weighed, the moisture allowed to evaporate in an oven at 100 C to dryness and then weighed. Corrections are then made to determine the amount of oven dry weight of the sample of which the spores or cells giving rise to the colonies on the plates were part.

B. Isolation and Culture of Fungi

Media Preparation

From the beginning of the study of molds in polluted waters and sewages, the Martin modification of the Waksman soil mold plate count agar was used both because the results described by Martin (1950) and because of the results obtained in this laboratory based on comparisons with other modifications of isolation media and substitutions of dyes, antibiotics and media ingredients. These comparisons were made in a series of 8 isolation experiments using materials from the Dayton, Ohio, sewage treatment plant, and samples from the usual series of stations on Lytle Creek, Clinton County, Ohio. This series of experiments was described in detail by Cooke (1954a, c).

The medium which was finally chosen for use in the isolation of soil fungi from sewage and polluted water was:

Dextrose	10.0 g
Peptone*	5.0 g
KH_2PO_4	1.0 g
MgSO_4	0.5 g
Agar	20.0 g
Water	1000.0 ml
Rose Bengal	0.035 g (1:30,000)
Aureomycin	30.0 $\mu\text{g/ml}$

*A study of the formulae of the composition, so far as known, of the various dehydrated peptones available from Difco and BBL showed that in so far as nitrogen content went there was little difference between phytone, soytone, and neopeptone. All of these peptones have been used satisfactorily.

It should be noted that as a result of observation of plated samples it became apparent that the KH_2PO_4 and MgSO_4 were unnecessary. This observation was backed up by analyses of peptones and listings of impurities in other materials used in media preparation. Because there were, apparently, enough impurities of potassium, phosphorus, magnesium and sulphur in the medium without adding these two chemicals their addition was discontinued after the first two or three years of isolation work. In the meantime, Difco and BBL added Cooke's Rose Bengal agar to their lines of dehydrated media. While these prepared media are adequate, one contains a rather high amount of peptone in comparison with sugar, the other contains more mineral salts than necessary.

Media used in plating the samples may be prepared the day before the samples are collected. In some laboratories the most convenient

method of storage is in 10 ml lots in culture tubes with metal closures in culture tube racks in a hot water bath ($49-55^{\circ}\text{C}$). Instead of this, the medium may be placed in agar storage bottles and kept in the refrigerator or on the laboratory shelf. If the latter technique is used the medium should be remelted just before use. In this case the antibiotic, the sample and the agar are pipetted into the Petri dish separately and the dish swirled to assure mixing of the three components.

Plating for Soil Molds

Probably the commonest fungi to be found in polluted waters of all types are those elements of the geofungi which are able to tolerate the changed conditions resulting from the pollution, together with those elements who merely wandered into the habitat, and are able to survive the journey through the pollution-influenced area. On this basis, the greatest amount of work in isolating fungi from samples of polluted waters is concerned with geofungi.

For the best results in the reading of plates, each plate should contain 40 to 60 colonies of fungi. To accomplish this, the sample must be diluted before it is plated. The extent of dilution for each sample can be judged after some experience has been gained in plating the different types of materials obtained. In general, liquids may be diluted only 1:10, although some cleaner waters will need no dilution. Plating techniques do not permit concentration of samples so that a 1:1 dilution is the least which can be carried out. A fairly rich sewage may be diluted 1:100; sludges containing about 4-6% dry matter, and relatively

poor clayey soils, 1:1000; and richer, fairly dry materials (dry matter 30 to 60 percent), as much as 1:10,000.

When the sample reaches the laboratory, a series of dilution flasks is prepared. In the first flask 135 ml of distilled water is placed, and in the second, third, fourth, and other flasks in the series, 45 milliliters. Fifteen milliliters of the sample, regardless of the type of material, are then added to the first flask. This will be a 1:10 dilution for any of the samples to be tested. For plates to be poured at a 1:10 dilution level, material from this flask will be used. When all the 1:10 dilution flasks have been prepared, they are placed on the rotary shaker and shaken one-half hour at 120 to 150 oscillations per minute to at least partially break up the particulate matter. Meanwhile other duties in the laboratory leading up to the final operation can be completed. Where convenient, the sample at this dilution can be placed in a blender at high speed for a minute or two. Probably damage to living cells is minimal and some particulate matter may be more effectively broken up than in the more gentle shaking procedure.

The flasks are removed from the shaker, and 5 milliliters of the 1:10 dilution are pipetted to the second flask in the series for a 1:100 dilution. This flask is shaken by hand to disperse the sample; if a third and fourth dilution is required the process is repeated by adding 5 milliliters of the 1:100 sample to the third flask for a 1:1000 dilution, and 5 milliliters of this to the fourth flask for a 1:10,000 dilution.

When a type of sample is studied for the first time, two dilutions may be plated out to compare the results of a higher and lower dilution. Then the dilution that gives the more adequate idea of the fungal content of a sample is chosen and that dilution is used for other samples of the type represented by the collection from which the series of dilutions was made. Of course, it is possible that the first sample from a location may give a high count and the second a low count. It may be that a 1:100 dilution will seem adequate once but the next time the plates will be too crowded for adequate counting. These difficulties, if not anticipated, can only be corrected by continued trial and error sampling and testing techniques.

Each sample is plated in replicates of five, and total counts obtained in the final count should be corrected accordingly. To plate the sample, five tubes of neopeptone - dextrose - rose bengal agar are removed from the water bath. To each tube 0.05 milliliter aureomycin solution (prepared by adding 1 gram Aureomycin hydrochloride to 150 ml distilled water; this solution can be stored in the refrigerator and is membrane filter sterilized prior to each usage) is added, plus one milliliter of the sample at the dilution required. The contents are immediately poured into 9-centimeter glass, or 10-centimeter plastic, Petri dishes and swirled. The top plate on each stack of five is labeled with a glass marking pencil giving sample number and date of inoculation. The samples are incubated at room temperature and light conditions for one week.

Other sets of plates can be prepared at the same time to determine the effectivity of this medium in comparison with others. Acidity can

be adjusted, the rose bengal may be omitted, the nutrient levels may be adjusted, other antibiotics may be tried, or other types of nutrients may be used.

After incubation the soil molds on these plates are ready to be "read". Two types of information can be obtained from them: the total number of mold colonies produced by this technique; and the number of colonies of each of the species of molds that were recovered by this technique from any one habitat. To correlate the numbers of mold colonies recovered from each sample with those from other samples, a uniform basis of estimate is necessary. The oven-dry weight of the sample has been chosen as this basis. The percentage of dry matter in the sample is obtained as described above, and for comparative purposes the number of colonies of fungi in each sample can be estimated.

If a plating technique is needed merely to determine the types of organisms associated with polluted habitats and no quantitative data are required, the above step may be omitted and a smaller sample used. Other types of isolation techniques described below give only qualitative data usually; there is no known technique for expressing the results quantitatively. This does not mean that the organisms are not important in the habitat.

Culture Techniques for Other Fungi

In addition to the geofungi, many species of which can be obtained by the plating procedure outlined above, other fungi are probably present in the sample. These may also be geofungi but their relationships may lie

more with the aquatic fungi, or their nutrient requirements may be more fastidious than those recovered on pour plates. The following techniques can be used to obtain some of these. The use of additional techniques may yield still other species the importance of which cannot be understood until the species are found, identified, and studied.

Sufficient dilute sample is removed from the 1:10 dilution flask to submerge and float two or three sterilized, halved hemp seeds in a Petri dish. If enough original sample was collected a portion of it may be added. This is incubated at room conditions of light and temperature for 1 to 2 weeks (3 to 4 days for some species). The true aquatic fungi, if present, will form colonies on the hemp seeds. Suzuki (see above) thought that each filament arising on a hemp seed resulted from the germination and growth of one zoospore. If this is true, and is borne out by additional experimentation, then this technique can be used for a quantitative estimation of the aquatic fungus population of a sampled habitat.

Hemp seeds on which colonies of aquatic fungi have become established may be transferred to fresh hemp seed cultures prepared as follows: 40 grams of activated carbon are added to a liter of sterile distilled water or lake water in a flask, and boiled. The carbon is filtered off, the water is sterilized in an autoclave, and dispensed at the rate of about 20 milliliters per Petri dish. Two or three sterile, halved hemp seeds and one of the hemp seeds from the original culture are added. To reduce or eliminate types of life other than the aquatic fungus, and to get pure cultures of single species of aquatic fungi, free floating

single hyphae are transferred to plates of corn meal agar. If contamination or mixture is too extensive, portions of the original colony may be planted on the corn meal agar and when growth occurs hyphal tips may again be floated with hemp seed in sterile carbonized, distilled or lake water. This is done by removing agar blocks with the desired hypha and placing them with the hemp seeds.

Some geofungi may be isolated on the hemp seed. These may be removed carefully to agar in Petri dishes and purified. It may be simpler to preserve some records of this type merely by making a permanent or a semipermanent slide of the colonies that appear.

To determine whether or not keratinophilic fungi are present in a sample of sewage, sludge, soil, polluted water, or even clean water, keratin-containing materials such as pieces of human skin (from sunburn peelings), human hair, horse hair or wool cloth are added to culture dish samples prepared as for hemp seed sampling. Skin and hair may be placed in ether overnight to remove excess fatty material before use. The molds that grow on these types of bait should be observed and transferred to suitable media such as Sabouraud agar, with or without horse hair or other baits.

A highly specialized group of organisms, the "aquatic Hyphomycetes" occurs in vernal ponds, in pools in streams, in roadside ditches, and in similar aquatic habitats. To determine whether these fungi are present in the habitat under study, pieces of partially or completely skeletonized dead leaves lying on the bottom of shallow pools, or samples of scums or foams from the surface of a stream near the bottom of a riffle, should be

examined under the microscope for the peculiar spores of these fungi. Spores may be observed and species identified from such examinations. With care, spores may be isolated and planted on agar to produce colonies of molds, which may be studied further for additional types of information.

On rare occasions species of predacious fungi have been observed in polluted waters either as invaders or as actual residents. These are predators of nematodes and were either isolated on hemp seed or observed as growths on samples of water from treatment systems. In one series of experiments, predators of rotifers were found in raw sewage with which activated sludge had been inoculated; the fungus developed such a voracious appetite that the success of the experiment was endangered. Such occurrences can be observed on occasion, but the responsible fungi are rarely or never obtained in routine cultural procedures such as those described above. To maintain them in pure culture, a culture of the animal used as food by the fungus is required.

Cultural Search for Yeasts

In addition to those colonies which appear on agar plates, yeasts may be recovered by the following technique. Two different media are used, the basic ingredient for both being Wickerham's yeast-nitrogen base (YNB-Difco) at the rate of 6.7 grams per liter. For each sample to be tested a pair of 250-milliliter Ehrlemeyer flasks is prepared, each with 50 milliliters of different media. To the first flask, 1% glucose is added; to the other, 20% glucose. The YNB and glucose components of the media should be filter-sterilized separately to prevent

heat breakdown of YNB components and caramelization of the sugar. To the media in the flasks 1 milliliter of the 1:10 dilution of each sample to be tested is added. The flasks are placed on a rotary shaker at 120 to 150 oscillations per minute for 64 hours. At the end of that time they are removed and allowed to stand on the laboratory bench for about 4 hours. The yeast cells, when present, will settle to the bottom, the bacteria will remain in suspension, and filamentous fungi will remain in suspension or float on the surface. A ring of filamentous fungi, yeast, and bacterial growth will develop at the point on the flask wall reached by the medium in the shaking process.

Sufficient plates of yeast extract-malt extract-glucose agar should be ready to inoculate two or three plates from each flask. The flask is tilted so that a concentration of yeast cells appears at the junction of the liquid medium line and the bottom of the flask, and a loopful of cells is removed; these are streaked onto the whole surface of the agar plates. Incubation is at room conditions of temperature and light for 2 to 3 days. If colonies are discrete they are removed to slants; if not, they are streaked on 15 % Diamalt agar plates. Cultures are purified by repeatedly streaking on Diamalt agar plates; the plates are observed daily to determine pickable discrete colonies. Cultures are carried on stock medium in slants until time is available for further processing. This is not a quantitative technique.

Filamentous fungi obtained during incubation of the flasks may be removed to plates of neopeptone-dextrose agar, pure cultured, and noted or picked for later identification. No quantitative data on filamentous fungi are obtained in this way, but the technique could indicate

something of the abundance of a species responsive to this particular type of isolation procedure in a particular habitat on a certain date. Mycelial balls, ribbons, and mats, grown in liquid shaker culture, may be broken up by dipping a knife in 95 % alcohol, burning off the alcohol in a flame, and cutting off chunks from the mycelium or ribbons of growth.

C. Isolation of Fungal Colonies

Separate colonies of molds or yeasts may be isolated directly to agar slants. A quantity of tubes may be prepared in advance. Stock cultures are maintained on one of several types of media; some use potato-dextrose agar, a semisynthetic medium which is somewhat different with each batch of potatoes used, others prefer Leonian's medium agar, a reproducible medium. In this laboratory I have used neopeptone-dextrose agar, a semisynthetic medium which is relatively constant in successive batches, while special groups of fungi can be maintained on special media to which each is more or less nutritionally adapted. With an L-shaped nichrome wire needle a portion of a discrete colony of a filamentous fungus or a yeast is removed and planted or streaked on the agar slant; cotton stoppers rather than metal closures are used. The tube is labeled, and it is stored for study after appropriate growth has taken place.

Isolates of all the species that appear on the primary isolation plates are never obtained, but isolates of most of them can be obtained with reasonable care. At first, the person reading the plates will record one or more colonies of a certain type of mold or yeast, and he will pick one of these colonies for future study. As familiarity

increases, he will be able to record certain types of molds without picking colonies. Such familiarity never accompanies the yeast colony; there are always only red and white yeasts and each different colony type should be picked and studied. In certain cases a mold colony may be made up of two species, a situation which can lead to confusion if the two species are not sorted out carefully at the beginning. A laboratory may not wish to maintain a large series of cultures, but may keep on hand a few cultures representing the different species that are found.

The study of the true aquatic fungi may be confined to the larger species easily observed on hemp seeds; if a more intensive study of this segment of a population is desired, then the smaller fungi associated with the larger ones as parasites of fungi and algae, symbionts, etc., may be studied, but the person carrying out this study should learn techniques from a specialist before becoming too deeply involved with these interesting fungi.

The isolation of pure cultures of yeasts and molds appearing in the shaker flasks requires a little more effort, as several species may grow in the same plate or slant for several transfers. Careful dilution and isolation procedures are required to separate these.

Those species of filamentous fungi and yeasts that are isolated by the above techniques may be identified from any of the several texts available for students on the classification of mold and yeast fungi. For preliminary screening of cultures, however, the descriptive information in my Laboratory Guide (1963) may be useful in identifying many of the isolates obtained.

D. Identification of Isolates

For most filamentous fungi, knowledge of spore-bearing structures is sufficient information upon which to base an entry into the literature. These species are identified on the basis of their morphology and even at the subspecific level physiological tests are not required. In order to get best development of spore-bearing structures some species groups need to be grown in special media. For Penicillium, Aspergillus, Paecilomyces, and similar fungi, best development of necessary structures occurs on Czapek agar. True aquatic fungi are best grown on hemp seeds, dead flies or other insects, corn meal agar, or some other material suggested in the literature of that group of organisms.

Because filamentous geofungi have easily disrupted sporiferous systems, the best way to observe them with least disturbance is in the slide culture. There are two types of these. Ten milliliters of agar containing nutrients on which the fungus grows best are poured in a Petri dish and allowed to harden. The solidified agar is then cut grid-wise into a set of squares somewhat smaller than the size of a cover slip. Petri dishes have been prepared in the bottom of which V-shaped pieces of glass tubing are placed and one or two glass slides are placed on the V-pieces. These are heat-sterilized or autoclaved in Petri dish cans or in wrapped packages. In the center of each slide is then placed a square of agar, a needleful of spores is taken from a culture of the fungus to be studied, and drawn around the four sides of the agar block. A sterile cover slip is added, 10 milliliters of sterile distilled water are pipetted into the bottom of the Petri dish, the dish is covered and allowed to

incubate at room conditions of temperature and light. After 3-4 days growth should become evident on the agar block. This is usually determined under the microscope. For rapidly growing species less time is required than for slow growers. When growth is in good condition for study it may be mounted in a drop of lacto-phenol-cotton blue.

Phenol (crystals)	10.0 g
Lactic acid	10.0 g
Glycerin	20.0 g
Cotton blue	0.05 g
Distilled water	10.0 ml

This is prepared by warming the phenol crystals in water in a beaker in a water bath until dissolved. Lactic acid and glycerin are added and then cotton blue or other stain as required for staining cell cultures for mounts.

Two mounts may be made from one slide culture. For the first take a clean slide, place a drop of mounting medium in the center, remove the cover slip from the agar block in the slide culture dish and place it with the side on which the growth occurred on the drop of mounting medium. Depending on the size of the dropper and cover slip more than a drop may be needed. The cover slip should never be disturbed to avoid disarranging the growth, sporophores and position of spores in relation to the sporophores. The growth on the slide may be mounted by removing the block of agar carefully, placing a drop of mounting medium in the center of the space occupied by the agar and carefully covering with a cover slip. Air bubbles may be removed by placing a drop of 70 % ethanol on the area of growth being careful to cause as little disturbance to the growth as possible. Before the ethanol is completely dry the mounting medium may be added.

These mounts may be made semipermanent by allowing the water in the mounting medium to evaporate for several days, then ringing the coverslip with clear nail polish.

Morphology is only part of the information needed on which to base an identification of yeasts. A number of physiological experiments are required to complete the required information. For these experiments a supply of fresh cells growing in bulk on 15 % Diamalt in 2 % agar is necessary. Ring and pellicle formation as well as cell measurements are made in 15 % Diamalt broth. For determination of growth on basic carbon and nitrogen sources auxanograms are prepared on YCB and YNB agar. For carbon auxanograms add 2 ml of a suspension of yeast cells, 1 ml of a 10X YNB solution (6.7 g YNB in 100 ml distilled water, filter sterilized), and 10 ml of 2 % agar, and swirl. After the agar is hard place a few crystals of glucose in the center and at the cardinal points place a few crystals of galactose, sucrose, maltose, and lactose. For nitrogen auxanograms prepare a YCB solution and cell suspension agar in the same way and at three equally spaced points on the surface at the rim of the plate after the agar has hardened, add a few crystals of ammonium sulphate, sodium nitrate and potassium nitrite. Growth in the agar under any of these C and N sources is recorded on a special note sheet on which growth results will be recorded together with morphological information.

To determine the ability of each strain of yeast to ferment sugars a yeast-autolysate solution is prepared and dispensed in 16 x 150 mm culture tubes in which inverted shell vials 8 x 30 mm in size

have been placed. The sugars glucose, galactose, sucrose, maltose, lactose and raffinose are used, the last 5 only if glucose has been fermented in a preliminary test. The medium contains 0.5% yeast autolysate and 2% of the sugars except raffinose which is tested at 4%. If raffinose is fermented a test should be made of melibiose.

To test the ability of yeasts to produce their own vitamin supplies, prepare a solution of vitamin free yeast base (VFYB) at the rate of 16.7 g in a liter of water, filter sterilize and dispense in culture tubes. Inoculate with the yeast strains to be tested and incubate at room conditions for a week. Degrees of cloudiness will represent varying abilities of the strains to grow in a medium without vitamins. Usually growth is fair to good the first time in this medium because of residual vitamins in the cells. Prepare a second VFYB solution and inoculate into it those yeasts which grew in the first from the growth which occurred in those tubes. If after the end of a week's incubation at room conditions growth is found, those strains are shown to be able to produce their own vitamin supplies, the others are dependent on their environment for vitamins.

To determine if yeasts will grow using ethanol as a sole source of carbon, prepare a YNB solution to which 1% ethanol is added, filter sterilize and dispense in 25 x 150 mm culture tubes. Inoculate with the yeast cultures, determine growth as an index of cloudiness and pellicle formation.

To determine the type of growth and whether pseudomycelium is formed, prepare some potato dextrose agar from fresh potatoes, and prepare

slides for a slide culture test. To the sterile slides on V-tubes in the Petri dishes add sufficient potato dextrose agar to cover the slide. After this has hardened, using a flattened nichrome wire needle inoculate two rows of cells on the slide and cover the central portion with a sterile glass coverslip. Growth under the coverslip should be modified by reduced amounts of oxygen available to it. The type of pseudomycelium, if any, mycelium, and budding is recorded by drawings on the note sheet.

Cell size and type of thallus are recorded from 3-day old growth in liquid wort (Dismalt broth) by measurements and drawings. Growth, pellicle and ring formation are recorded after 3 and 30 day's growth on liquid wort.

To determine the ability of a yeast culture to assimilate a variety of carbon sources, strains and media are prepared for replication in the Wickerham series of carbon sources. There are 34 compounds in this series of which two are infrequently used because of availability. YNB and carbon sources are prepared separately and in most cases each is filter sterilized. The YNB is prepared according to the 10X formula mentioned above and on the container. Flat bottomed Petri dishes are used, the YNB, the carbon source and the agar are prepared separately, added to the plates separately and swirled to mix. After the plates are poured the agar is allowed to harden and the surface to dry overnight. Yeast cell suspensions are prepared in 8 x 30 mm shell vials 2/3 filled with distilled water and autoclaved. The aluminum foil capped vials are inoculated with the yeast cells with a loop and transferred to the replicator base in sequence. Using a replicator guided by

metal poles, or a similar arrangement, all 25 strains from one block of vials are inoculated onto each carbon source plate in rapid succession. An extra plate may be inserted in the series for use in 37 C incubation studies. Other plates may be included if necessary. The replicator plates are read at the end of 3, 6 and 14 days of incubation at room conditions of light and temperature.

In occasional cases a strain will produce ascospores in one of the tests mentioned above. For those which do not there are several types of media to which the cultures can be exposed for possible ascospore production. These are described in the yeast literature, as well as in the Laboratory Guide (Cooke, 1963).

Data accumulated on the data sheets are summarized by using them in the attempt to place a strain with its proper species through the use of the yeast manual (Lodder & Kreger-van Rij, 1952), the supplemental literature, or the revision of the manual to be published shortly.

E. Yeast Enumeration

It has been indicated above that enumeration of fungus components of the population is based on a plate count procedure. From colonies appearing in pour plates from dilutions made from known amounts of soil, stream bottom materials, waters, sludges, and other types of samples, counts are made and corrected so that the final amount will correspond with that estimated for the dry matter in an equal amount of sample.

In the sampling of populations of organisms in clean and polluted streams, and in several types of sewage treatment systems, yeasts, as well as filamentous fungi, were obtained. Numbers of yeast colonies

appearing on agar plates were low. L. J. Wickerham, Zymologist, Northern Utilization Research and Development Laboratory, U.S.D.A., suggested a shaken flask enrichment culture technique for determining the relative incidence of yeasts, but in earlier work no quantitative evaluation of the technique was made. It has been used in routine surveys, and qualitative information concerning the distribution of more than 90 species of yeasts has been accumulated for 10 years.

As a result it became apparent that, as the organic enrichment of a stream or other body of water, or even of soil increased, the number of yeast cells increased as well as the number of yeast species. The problem now was to demonstrate the number of cells present in a sample. Lund (1954) and di Menna (1960) used a plate count method. Batista, et al. (1961) reported presence of yeast species in terms of percent of numbers of samples or animals studied. Miller, et al. (1962) gave a qualitative report of species isolated in terms of "few, moderate or numerous". Alexander (1961) recommended the "most probable number" (MPN) technique (APHA, 1960) for estimating the populations of root disease and other fungi in the soil. I decided to use this method, as well as a modification of it, the "indicated number" (IN) technique, in which single cultures of a single dilution are used rather than replicates. It is less accurate but adequate for my purposes. The IN technique or Phelps test (Prescott, et al., 1946) is used more often in this work than the MPN technique because of space and time considerations; both methods have been used in connection with the media and techniques suggested by Wickerham. In discussing the MPN technique (APHA, 1960),

the statement is made: "It is also quite probable that the growth in a tube may result not from one but from many organisms. It is more convenient, however, to assume that growth develops from a single individual". The same line of reasoning applies to results from the IN test.

It should be noted that the YNB (yeast-nitrogen-base)-flask technique is an enrichment procedure for yeast development. As the nitrogen in the ammonium sulfate is assimilated increasing amounts of sulfuric acid tend to inhibit bacterial growth. However, certain bacteria are able to develop in this medium, especially when yeasts are few or absent. The 20% glucose is also inhibitory to most bacteria in the sample.

Methods

Samples of soils, sewages, sludges, waters, and other materials are suspended in distilled water at a ratio of 1:10. This suspension is shaken on the rotary shaker at about 150 oscillations/min. for about a half-hour.

The nutrient solution is prepared with YNB as recommended by the manufacturer. YCB (yeast-carbon-base) can also be used but nitrogen and carbon sources must be adjusted to complete the medium. All nutrient solutions are cold sterilized by passage through membrane filters. They are dispensed in 50-ml lots in sterile 250-ml flasks. One flask has 1% glucose as the carbon source; another set, 20% glucose. For routine surveys for qualitative description of a population, 1 ml of the 1:10 dilution of the original sample is added to one flask of each medium

and these are placed on the shaker for 64 hours. When the IN method is used, 1 ml of the dilutions 1:10, 1:100, 1:1000, 1:10,000 and 1:100,000 is added to one flask of each nutrient medium. The IN flasks are shaken for 64 hours. Using the MPN method, since sufficient shaker space is not available, 25 ml of nutrient are added to 25 x 150 mm culture tubes. One ml of each dilution is added to each tube in replicates of 5 for each medium. The tubes are incubated for 7 days on the laboratory bench without shaking.

After incubation the IN flasks are removed from the shaker and placed on the laboratory bench. After 4 hours the yeast cells have settled to the bottom of the flasks. Since no antibiotics were used, and the pH was unadjusted, some bacterial growth may have occurred. Filamentous fungi including species of Alternaria, Aspergillus, Aureobasidium, Fusarium, Geotrichum, Mucor, Penicillium and Trichoderma, may have developed, and balls or chunks of growth may rest on the bottom or chunks may remain in suspension. Sediment from the bottom of the flasks is streaked on pairs of yeast extract-malt extract-glucose agar plates. After 2-3 days the resulting growth is restreaked directly or from aqueous suspensions of cells onto the same medium or onto 15% Dismalt agar plates, and individual colonies are picked. Colony mixtures may need to be streaked several times before pure cultures of most species are obtained.

Bottom growth from the MPN tubes incubated on the laboratory bench is also streaked on agar plates for species identification. Different species groups will appear in the shaken and still-culture

vessels. In addition, in the still-culture tubes mold growth may present isolation problems since it may mask the yeast growth making it difficult to get representative streaks on the agar plates. For analyses, such as turbidometry with the Klett-Summerson photoelectric colorimeter, mold on the surface of the incubated tubes is a drawback. In the suspension to be used for turbidmetric determinations, mold spores and mycelial fragments may contribute larger sources of error than the bacterial cells obtained on the shaker. Molds also mask or prevent film-yeast growth. An additional source of variation is the availability of oxygen to yeast growth in the bottom of the tube.

A minimal IN or MPN count may be obtained from growth in the flasks, or from the first plates on which the growth from the flasks was streaked. Thus, if yeasts appeared in the flasks receiving 1:100,000 dilution, at least 100,000 yeast cells may be presumed to have been present per ml of original sample. But if two different forms, e.g., red and white yeasts appeared, at least 200,000 cells were present. If no growth appeared in the highest dilution, the next highest dilution in which yeast growth appeared would become the source of the reporting value.

If, in addition to the minimal number of cells represented by any one of the flasks, one wants to know the species of yeasts, colonies must be picked from plates that were streaked from all dilutions. The reason is that some species may be present in low numbers and some may be present throughout the series.

If eight samples from different stages in the operation of a sewage treatment plant were being processed, five dilutions were made

of each sample, and each dilution was inoculated into two different media, 80 flasks would have been incubated on the shaker and their yeast populations (assuming growth occurred in each flask) streaked onto 160 plates. Despite considerable duplication, as many as 150 isolates may be available for species identification. Yeast identifications are then initiated and may be carried on in a mass production system. It is not safe to base an identification on an incomplete series of tests.

Counts are based primarily on enrichment in YNB with 1% and 20% glucose. For certain sets of samples other media yield additional species, thus making population analyses more complete, if not more complex. If species other than those isolated in the two described media are present in a sample they may be isolated on pour plates of neopeptone-dextrose-rose bengal-Aureomycin agar, as described above, or on other types of enrichment media as yet untried for the sewage and otherwise organically polluted habitat. Species of at least Cryptococcus, Rhodotorula, and certain Trichosporons do not grow as readily in the shaken-culture technique, in the enrichment media used, as do species of Candida, Torulopsis, other Trichosporons, Kloeckera, and sometimes Hansenula and Saccharomyces.

In the routine processing of samples, when only the 1:10 dilution is used, a count of yeast cells in the sample cannot be reported since higher dilutions may have yielded yeast growth. Therefore, only occurrence of yeast species is reported from sampling of this type. However, as much care is taken in all cases in the picking of colonies for

identification since, in general, at the primary isolation level, all red yeasts look alike, and most species of white yeasts have similar appearances.

7. Special Considerations on Enumeration

Throughout my studies on the occurrence of fungi in polluted water and sewage, I have used a quantitative technique in which the number of colonies of a fungus appearing on an agar plate, or the total number of colonies of fungi on these plates has been the reporting method. I have advocated the use of this type of technique for describing populations in the Laboratory Guide and by implication in my reports and papers.

N. A. Burges (1965) has shown that the use of plate counts in the study of soil fungi permits the reporting only of spores in the soil which have survived the conditions in the soil and in the plating procedure. He has pointed out that the technique does not account for the large amount of active mycelium in the soil which may ramify throughout, occur in aggregates of soil particles, and which do not grow under the conditions of the plating procedure. It is also quite possible that the dye rose bengal inhibits the growth of mycelial fragments or filaments of any size.

The growth of fungi in sewage and sewage-polluted waters may be thought of as being equivalent to growth of fungi in a liquid medium. There sporulation is usually atypical morphologically speaking, if it occurs at all. Sporulation may occur in sewage and sewage-polluted waters but sporulation is atypical and not as abundant as in dry soil habitats. It is suggested that within the two types of habitat, normal

soils and organically enriched waters and wet soils, there are differences in the adaptation of the fungi to the nutrient supplies. In the former the nutrients are received sporadically under conditions wherein moisture is not always readily available so that growth in the presence of nutrients is sporadic. In the latter, nutrients are received by the population continuously, moisture or an excess of moisture is always present and growth can take place continuously. Where there is continuous growth, in many cases, there is little need for the development of a resting stage and sporulation is weak, if present at all.

On the above basis, in the plating of organically polluted water or wet soils, the only interruption to the growth process, which may be largely superficial rather than partly embedded in soil particle aggregates, is in the taking of a sample, the conditions under which the sample is transported and stored, and the conditions of the plating process. In the plating and incubation processes the principal inhibitor to mold growth could be the presence of rose bengal. It has been noted in some cases that when rose bengal is left out of the media formula, more colonies, but not necessarily more species, appear on the poured plates in the presence of aureomycin.

A second factor contributing toward a bias in the counting of mold colonies on agar plates is found in the nature of the fungus itself. If we are studying a sample of material in which the fungal growth forms a continuous mat over a surface, the mat must be broken up in order to approximate an even dispersal of cells through the isolation medium. The mat includes mostly filaments of growth of several species of fungi.

In some cases the filaments will be composed of cells which do not break apart readily but which may have a ratio of length to width of 2:1 to 10:1 or may be even longer. Other species may have a mycelium which breaks apart readily, on disturbance, to individual cells; and the yeasts are found as clumps of individual cells which may or may not be washed apart in a shaking process. Simple shaking without abrasives will not break up the mat efficiently. If the fungi have been growing through a sandy soil, in the mud and sands of a creek pool, riffle, or bank, the breaking process in relatively gentle shaking will be more efficient. Slimes growing on glass slides may be broken up in a blender. However, in no case will the final result equal the perfection required if every cell in a given slime mass were made available as a viable unit. In the case of all species which do not readily break up into spores or which are not colonial clumps of cells, the best which can be expected is a rupture of the mycelium so that alternate cells would remain viable, the ones between having been ruptured to ensure the break.

If one were to use percentage occurrence of species in a sample based on plate counts such as those described above, he would fall into the same trap since the numbers obtained on which the percentages would be based might not be the result of an accurate technique.

The use of multiple techniques for obtaining fungi from samples can yield a variety of species not obtainable from the use of a single technique. In cases where plate counts, shaken flasks, hemp seeds and other baits, and still-liquid cultures, have been used for the same

series of samples, it has been found that more than twice as many species of fungi have been recovered from a single sample than on the most productive of any of the single techniques. If the percent occurrence of a fungus in a sample were obtained from this type of multiple sampling, and the number of media to which it reacted or not were known, an index of the importance of a fungus in the sample might become available.

Table 2 summarizes the number of observations of fungus colonies on plates or preparations of all types made during the period of this study, 1952 - 1966.

Table 2. Numbers of fungi observed in tested samples

	Observation Number*
Sewage Treatment Plants or Systems	
Dayton Sewage Treatment Plant, Regular Series	4636
Continuous Sampling Series	1885
Miscellaneous Trickling Filter Plant samples	385
Activated Sludge-type plants, all samples	1166
Sludge Digestion Experiment	3035
Sewage Treatment Plant Sludges (digestion)	1847
Lebanon Waste Stabilization Ponds	7662
Miscellaneous sewage treatment plant samples	607
	<u>21223</u>
Polluted Streams	
Lytle Creek	4496
Duck Creek	198
Berry Creek	561
Cache la Poudre River	1500
Cache Valley - Bear River	1475
Acid Mine Drainage streams	6640
Miscellaneous stream samples	827
	<u>15697</u>
Soils	
Spent Sulphite Liquor Disposal, Michigan	785
Alaska Glacier Recession samples	702
Miscellaneous soil samples	1365
	<u>2852</u>
Miscellaneous Environmental Series	
Mattress Samples	1855
Air Spora samples	1300
Miscellaneous samples	453
	<u>3608</u>
Total Fungus Observations	43,380

*Observation Number refers to a value determined by the number of times the fungus was observed in the series of platings of samples studied. In the case of pour-plates it refers to the number of colonies observed. In the case of shaken flask cultures it refers to the number of isolates studied. In the case of the continuous sampling study it refers to the number of weeks a species was noted regardless of the number of colonies such notation represented.

It will be noted that 85% of the observation numbers are based on samples obtained in the study of sewage treatment systems and polluted stream waters.

III. Aquatic Fungi *

True aquatic fungi have been known for 135 years, but work with them during that time has centered around three fields of interest. Early observers noted that certain aquatic fungi grew around dead animals in the water such as insect larvae, flies, and other mature insects, and that other types of fungi grew on living fish. Later it became apparent that one or two species of aquatic fungi were associated with polluted waters in streams, sewer outlets, wastes from certain specialized industries such as sugar factories, and even on trickling filters in biological sewage treatment plants. Since the latter part of the nineteenth century there have been several mycologists searching intensively for aquatic fungi. As a result of these efforts a substantial body of literature has been developed around the aquatic fungi which are found in relatively clean waters including lakes, pools in streams, and ponds. These collectors have not similarly studied the populations of polluted waters, possibly because this habitat is unattractive to the aesthetic senses. Accordingly, there is only a limited literature on aquatic fungi found in polluted waters.

In 1951, Harvey (1953) collected from streams throughout the Little Miami River Basin in Hamilton, Clermont, Warren, and Clinton Counties, Ohio, in a search for aquatic fungi. He chose portions of streams which could be considered relatively lightly to heavily affected by pollution as evidenced

* Reproduced by permission from Sewage and Industrial Wastes 31: 1316-1322. 1959. Part of this material was also used in Ohio Journal of Science 60: 144-148. 1960.

by the nature of the communities through which these streams passed. He found few, if any, aquatic fungi actually living in reaches of the streams which were heavily polluted.

From October 1954 to June 1958 an irregularly spaced series of tests was made to determine if it would be practical to assay populations of aquatic fungi for use as a parameter of water quality in stream pollution investigations. If a relatively rapid method could be found to demonstrate the aquatic fungus population of a stream, especially in the more polluted reaches, additional useful information could be made available to persons concerned with stream sanitation. At the same time the question of whether there is an aquatic fungus indicator of pollution could be answered.

Techniques

The technique used by Harvey was relatively simple. A sample of the stream consisting of water and stream bed materials was placed in a culture dish in the laboratory, and sterile, cracked hemp seeds were added. Within several days, if aquatic fungi were present, their zoospores would reach the hemp seed and there develop a colony in the form of a halo around the seed. This mycelium could be subcultured on fresh hemp seeds in sterile water in which carbon had been boiled and filtered off, or it could be subcultured on corn-meal agar slants.

In the studies reported here, other techniques were used, involving the use of bait. At first, the baits used were enclosed in cylinders made of 1/4 in. mesh hardware cloth, stoppered at each end with corks. From one of the corks a steel wire skewer on which a piece of beef, a piece of apple, a whole date, and a rose hip or hawthorne fruit were impinged,

projected into the cylinder. The cylinder, floating partially submerged, was tied to some more or less permanent object on the shore and left for a week. Then the baits were transferred to a container filled with water from the sampling location, returned to the laboratory, and placed in sterile carbon water with sterile cracked hemp seed. Another technique followed a suggestion by Hohnk and Bock (1954). Bags of loosely woven nylon cloth, containing sterile, cracked hemp seed, were tied to the shore of the stream being sampled, and left for three days. These bags were returned to the laboratory in containers partly filled with water from the sampling site.

In one series of tests, aluminum tea balls were prepared in the laboratory by adding cracked hemp seed, assembling three on a string, wrapping, and sterilizing. These assemblies then were sent to a field survey team working on a tributary of the Potomac River. Each tea ball contained three hemp seeds and a multiple unit as described was used at each of six stations. One tea ball from each site was removed from the water after 2-, 4-, and 6-day exposures. The tea balls, or their contents, were wrapped in absorbent tissue paper, then in absorbent toweling, the whole tagged and sent by regular mail to the Cincinnati laboratory. In most cases, water molds were recovered from these collections by the use of fresh hemp seeds.

On the basis of this experiment, tea balls were used for the remainder of the test series. They were allowed to remain in the sampling station for 3 to 5 days depending on work schedules of persons involved in placement and collection. Experience showed that the shorter period of exposure was preferable.

After a colony had developed on hemp seed for at least one, sometimes

two weeks, it was in a robust state actively producing many sporangia and sometimes oogonia with oospores. In this condition the relatively fresh cultures of saprolegniaceous fungi frequently were transported to other mycologists for identification or validation of identifications. Either cotton or folded laboratory tissue was moistened, a hemp seed with its colony placed on it, the tissue folded, the total wrapped in paper toweling, and numbered. When preparing cultures for shipment the primary wrapping material was damp enough so that it would not dry out in transit but not so wet that it would moisten the glue on the envelopes. The group of cultures wrapped in paper toweling could be wrapped again in wax paper or aluminum foil and this package could be placed in a box, culture tube mailing can, or envelope for mailing. On receipt of such material by cooperating specialists, to whom the collections were sent, the parcels were opened and planted again with fresh hemp seed. Usually, in the case of cultures wrapped in cotton, too many cotton fibers prevented immediate study, while those wrapped in tissue could be observed directly on being refloated in water.

Streams Sampled

To determine whether aquatic fungi can be found in grossly polluted waters, streams in the vicinity of Cincinnati were sought which would provide both clean reaches for control, and weakly to grossly polluted reaches for contrast.

Because there is a thorough background of biological knowledge on Lytle Creek, Clinton County, Ohio, resulting from intensive studies by others (1952) (1956), this stream was sampled twice. When baits were placed at the

eight stations used in the present studies, it was assumed that conditions were essentially the same as those reported earlier. However, when the second set of baits was removed it was discovered that the Wilmington, Ohio, sewage treatment plant had added activated sludge treatment to the primary plant reported on in the earlier work. This change accounted for the cleaner characteristics of the stream.

The village of Batavia, Clermont County, Ohio, discharges primary treated sewage into the East Fork of the Little Miami River. Two sets of baits were exposed in the river, one above the town and one below this treatment plant.

Raw sewage from Williamsburg, Ohio, enters the East Fork of the Little Miami River more than eight miles above Batavia. This sewage flows slowly from sewers at three principal streets. Baits were placed in pairs at three points along the stream: in clean water above the outfall, and in what may be termed "recovery zone," below each of two outlets.

Paired nylon bag samplers were placed at three points along the Great Miami River at Hamilton, Butler County, Ohio. One pair was placed in a relatively clean, quiet impoundment above a paper mill, another was placed below the waste outlet of the mill, and the third below the lower outfall of the City of Hamilton where raw sewage flows into the river. No positive results were obtained from this test.

A stream survey was in progress on the North Branch of the Potomac River in October 1956, and tea ball samplers were exposed here also. Six stations were sampled and water quality data for them are given in Table 3.

Table 3. River Water Quality Data, North Branch, Potomac River, Maryland, Oct. 1956

Station	BOD mg/l	DO mg/l	pH	Notes
6	1	Near Saturation	4-5	Acid Mine drainage.
7	-	-	-	Very clean, practically drinking water quality
9	20-40	3-7	-	High solids, COD, and alkalinity. Pulp and paper mill 1 mile upstream discharging untreated sulfate mill waste and sanitary sewage.
14	5-10	6-8	-	20 miles below station 9, still quite polluted. Small towns within 14 miles discharge untreated sanitary sewage.
16	10-15	3-6	-	6 miles below station 14. Rayon processing plant discharges untreated process and sanitary wastes 1.5 miles upstream.
19	8-10	3-7	-	8 miles below Rayon plant, below Cumberland, Md., but above sewage outfall.

Finally, tea balls were exposed on two occasions in Mill Creek, Hamilton County, Ohio, at five different points in the drainage system, including two relatively clean recreation lakes in County parks near the headwaters, as well as in highly polluted areas downstream. Exposures were made in September 1957 and June 1958.

Although referable to a somewhat different period than represented by the data from Mill Creek in Table 4 the water quality data in Table 5, which were made available by the City of Cincinnati, show the general character of three of the five stations. Such water quality data serve to complete the information necessary for full evaluation of a stream.

Problems experienced in the bait program were largely centered about curiosity of persons who frequented places where baits were exposed. However, such curiosity was expressed less frequently than expected, and baits were lost only on Lytle Creek and the Great Miami River.

Species of fungi found on the several surveys are listed in Tables 4, 6, 7, 8, and 9 for each of the streams studied. Where more than one bait was used, the same species of water mold was recovered on one or more of the different baits.

Discussion

When the aquatic fungi listed in Tables 4, 6, 7, 8, and 9 were studied in relation to the water quality conditions described, it becomes evident that there is little, if any, correlation between pollution aspects of habitat and fungus occurrence. Aquatic fungus populations in Lytle Creek at the times of sampling were relatively uniform in contrast to Harvey's findings indicating that the stream may have been relatively clean. No particular

Table 4. Fungus Species found in Mill Creek, Cincinnati, and Hamilton Co., Ohio*

Station**	<u>Achlya</u> <u>ambisexualis</u>	<u>Dictyuchus</u> <u>monosporus</u>	<u>Achlya</u> <u>sp***</u>	<u>Achlya</u> <u>oblongata</u>	<u>Achlya</u> <u>bisexualis</u>	<u>Aphanomyces</u> <u>parasiticus</u>	<u>Achlya</u> <u>dubia</u>	<u>Olpidiopsis</u> <u>fusiformis</u>
1	x	x	x	-	x	x	x	-
2	-	x	x	-	-	-	x	x
3	-	-	x	-	-	-	x	-
4	-	x	x	x	-	-	-	-
5	-	-	-	-	-	-	-	-

* Direction of stream flow from Station 1 to Station 5

** General description of stations as follows:

1. Upper end of Sharon Lake, Sharon Woods County Park: quiet water supporting surface growth of duckweed and subsurface growth of water and shore plants. Lake stocked with fish. On a tributary of the East Branch of Mill Creek. Pollution sources: farmyards and occasional septic tank overflows.

2. Upper end of Winton Lake, Winton Woods County Park: Quiet water supporting heavy bloom of a blue-green alga in the autumn. West Branch of Mill Creek. Pollution sources: Area residents report extensive tapping of septic tank overflows into tributary creeks.

3. Evendale, East Branch of Mill Creek: At crossing of State Route 126 (By pass U.S. 50). Bottom muddy, no apparent surface or subsurface water plants. Pollution sources: wastes from several industries including chrome plating wastes. Probably some sanitary sewage.

4. Carthage, main stream of Mill Creek: Crossing of Springfield Road, Ohio Route 4. Within Cincinnati limits. Stream flows over broad riffles and cement barriers formed from bridge abutment. Pollution sources: industrial wastes from additional types of industries including rocket and jet motor plants and paper mills.

5. Winton Place, Cincinnati, main stream of Mill Creek: At crossing of Spring Grove Ave. below Ivorydale. Stream flows over broad riffle-pool area, has just fallen over small cement barrier and within a few feet will receive sewage and wastes. Pollution sources: probably some industrial wastes enter the stream above this point but much of this material is now diverted to interceptor sewers.

*** Non-fruiting.

Table 5. Water quality data, Mill Creek, 1957. (Based on data supplied by the City of Cincinnati.)

Station	Coliform Count	DO mg/l	BOD mg/l	Water Temperature °C	Air Temperature °C	pH	Alkalinity mg/l	Turbidity units	Time of observation
3	23,000	9.6	-	7.8	(a) Mar. 20	8.3	290	25	1030
4	9,300	8.0	-	10.0	2.8	8.1	250	25	845
5	93,000	6.9	100.0	11.1	6.1	7.0	200	45	1300
3	93,000	9.7	2.9	10.0	(b) Apr. 17	7.9	260	25	900
4	750,000	8.4	17.3	11.7	12.8	7.7	230	60	1015
5	930,000	6.2	346.4	12.2	11.1	7.4	210	200	1130
3	230,000	7.7	3.8	15.0	(c) May 20	7.6	130	900	845
4	430,000	7.3	7.8	15.5	12.8	7.7	130	900	945
5	430,000	6.4	16.0	16.1	13.9	7.4	120	1,000	1045
3	43,000	4.7	1.8	22.0	(d) July 10	7.6	310	35	845
4	23,000	6.7	1.9	22.0	21.0	7.6	190	25	900
5	93,000	4.2	5.0	24.0	24.0	7.4	200	25	1015
3	93,000	7.2	3.1	21.5	(e) Aug. 22	7.8	300	25	1040
4	23,000	7.3	6.8	21.0	26.0	7.4	190	25	955
5	930,000	0.1	93.0	24.0	30.0	6.7	230	49	1205

Table 6. Fungus species found in Lytle Creek, Clinton Co., Ohio

Station	<u>Saprolegnia</u> <u>sterile</u>	<u>Saprolegnia</u> <u>delica</u>	<u>Saprolegnia</u> <u>ferax</u>	<u>Dictyuchus</u> <u>monosporus</u>	<u>Achlya</u> <u>sp.</u>
8.7	-	x	x	-	x
7.6	-	x	x	x	x
7.2*	x	-	-	x	-
6.5	x	-	x	-	-
5.2	x	x	-	-	-
4.2	x	x	x	-	x
2.8	x	x	-	-	x
1.0	x	-	x	-	-

* Savage treatment plant outfall; direction of stream flow is from Station 8.7 to 1.0

Table 7. Fungus species found in East Fork, Little Miami River, Batavia, Clermont Co., Ohio

Station	<u>Saprolegnia</u> <u>ferax</u>	<u>Dictyuchus</u> <u>monosporus</u>	<u>Achlya</u> <u>americana</u>	<u>Leptomitius</u> <u>lacteus</u>
Above Batavia	x	x	-	x
Below Batavia	x	x	x	-

Table 8. Fungus species found in East Fork, Little Miami River, Williamsburg, Clermont Co., Ohio

Station	<u>Saprolegnia</u> <u>ferax</u>	<u>Achlya</u> <u>americana</u>	<u>Achlya</u> <u>tutia</u>	<u>Dictyuchus</u> <u>monosporus</u>	<u>Olpidiopsis</u> <u>saprolegniae</u> var. <u>levie</u>	<u>Leptomitia</u> <u>lactea</u>
Above Williamsburg	x	x	x	x	-	-
In Williamsburg	x	x	x	x	x	x
Below Williamsburg	x	x	x	x	-	-

Table 2. Fungus species found in North Branch, Potomac River, Maryland*

Station	Achlya sp**	Achlya aer- icana	Achlya sp. nr. bisex- ualis	Achlya flage- llata	Achlya prol- ifera	Dicty- uchus anom- olus	Dicty- uchus mono- sporus	Iso- achlya sp***	Olpid- opsis sapro- legnis	Olpid- ium (on D. mono- sporus)	Pythium sp	Sapro- legnia sp. nr. flavida	Sapro- legnia sp. nr.	Sapro- legnia sp. nr.	Sapro- legnia sp. nr.
6	x	x	x	-	-	-	x	-	x	-	x	-	-	-	-
7	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	x	-	-	-	-	-	-	x	-	-	-	-
14	x	x	-	-	-	-	x	-	-	-	x	-	-	-	-
16	-	x	-	-	-	-	x	-	-	-	x	-	-	-	-
19	-	x	-	-	x	x	x	x	-	x	x	-	-	-	-

* Direction of stream flow from Station 6 to Station 19

** Non-sporulation

*** Non-fruiting

influence on water mold populations was noted in the East Fork of the Little Miami River either by the effluent from the sewage treatment plant at Batavia, or the raw sewage at Williamsburg. If any relation between pollution and the occurrence of water molds can be noted in the Potomac River, it is that aquatic fungi do not grow well in clean water. The downstream decline in occurrence of aquatic fungi in Mill Creek may be related to increasingly heavy loads of industrial wastes which may contain substances toxic to certain types of life.

In both the Potomac River and Mill Creek, accuracy and ease of sampling were affected by unexpected high water following rain which flushed out pools and transported planktonic life to points downstream. This had the effect of making the pools and the streams appear temporarily clean. The actively swimming zoospores of aquatic fungi are planktonic and thus could easily have been accidentally trapped by baits in other parts of the stream.

Relationships of aquatic fungi to dissolved oxygen and biochemical oxygen demand of a stream are as yet imperfectly understood. It is assumed that such fungi require relatively high levels of dissolved oxygen in the water although it is known that certain water molds are microaerophilic. Since no particular distribution pattern has been observed in relation to polluted reaches of several streams, no statements can be made in respect to oxygen requirements of the fungi listed herein.

An attempt was made to obtain chitrids that habitually grow on pollen by the use of goldenrod and aster pollens in the autumn and Douglas fir pollen in the early summer. However, possibly because of the short period

of time used for exposure, 5 and 3 days, respectively, no such fungi were obtained in the Mill Creek basin.

Summary and Conclusions

A number of relatively common aquatic fungi have been isolated from pools in areas of streams that receive domestic sewage and industrial wastes. Techniques of bait exposure are easily applied, but results of exposures made to date indicate that additional information gained in this way has little value in determining the degree of pollution of a stream. In part, this may be due to having used too short an exposure time to obtain certain types of organisms; in part, it is due to the scouring of flash floods from unpredictable precipitation. No aquatic fungi have been found with restricted distribution to polluted reaches of streams, and similarly no aquatic fungi have been found which are restricted to so-called clean water portions of streams. It is quite possible that more intensive study of the technique of using special baits, sampling periods, and altering other variables may yield a procedure of greater diagnostic value.

When making surveys of any type of body of water for aquatic fungi maintenance of a record of water quality data for sites at which aquatic fungi are found is recommended.

IV. Geofungi in Streams

A. Introduction

Throughout a discussion of the general biota of streams (Cooke, 1961), and of the special biotas of aquatic fungi, it has been stated or hinted that certain more prosaic types of fungi also occur in the aquatic habitat. These are the fungi with which the bulk of this report are concerned. They are without special organs of dissemination capable of searching out a special food supply, without special organs on the spores to aid in anchoring the disseminule or to resist the movement of water. These fungi reproduce, but poorly, by means of spores in an aquatic environment. If a piece of mycelium would come to the attention of the plankton or benthos specialist microscopically studying incidental samples, the type of spore-producing structures would be so atypical, as a result of having been produced in water, that the available keys, should they be used, would provide little assistance. If the spores of these fungi become embedded in accumulated foams, they would be readily overlooked, usually on the basis of size and recognizability. If colonies of these fungi develop in the same cultures in which chytrids or saprolegniaceous fungi are being studied, they would be discarded as contaminants, for the spores of many of these species are common in the air, especially of laboratories and other closed places.

On a habitat basis, we may consider the fungi which have been mentioned as belonging to two principal groups: 1. The hydrofungi, those species whose life cycle is adjusted to be completed in water, including water present only as a film sufficiently thick to permit the swimming of the zoospores or gametes. These, then, include the aquatic Phycomycetes of both the holocarpic and filamentous types, and the aquatic Hyphomycetes whose special branched or sigmoid spores are always produced under or on the surface of water.

2. The geofungi, those species familiarly known as the soil fungi, which are not especially adapted to an aquatic existence but whose life cycle may be completed under water because of an adequate supply of nutrients. The first group includes obligate aquatic fungi; the second includes facultative aquatic fungi.

According to Garrett (1956) the life cycle of a saprobic fungus living in the soil is as follows: a piece of dead organic matter such as a root, piece of leaf or stem, etc., comes to rest near a spore of a fungus. Activated in some way by the presence of this nutrient, the fungus spore germinates, the mycelium uses that fraction of the dead organic matter available to it, goes into a resting condition by producing a large number of spores, and the cycle is complete. In any body of water two types of nutrient materials are available to the fungi. The first is in the form of solid organic matter in suspension or settling out onto the bottom. Here, if such a fragment and a spore capable of using it are brought together, the cycle described above will become operative. However, dissolved in the water are other nutrients some of which will be readily available to the fungus. Upon germination of the spore, the fungus continues to grow indefinitely in the presence of this continual bath of nutrient materials, no matter how dilute. Depending on the supply of nutrients and the ability of the fungus to grow indefinitely, a small to large mass of mycelium may develop. Occasionally, this mycelium may sporulate, but such spore production may be atypical. In periods of flood, scouring action of inert materials in the stream bed results in the removal of much, if not all, of this growth. That not completely removed may be used to produce a new mycelial mat, that washed away may come to rest as a single mass or as many small fragments in other

parts of the stream bed or on the bank above the influence of the water. Depending on its ability to adjust to the new conditions of its existence, the mycelium may die, may go into a resting stage, or may continue to live, growing at the expense of dissolved, colloidal, or solid suspended organic matter in the water. Depending on such stream conditions as available nutrients, ability of existing spores to germinate, and of existing mycelium to continue growth, the fungus population will be small to large, yet the techniques of the plankton or benthos student will not demonstrate the presence of such growth to any extent. Fragmentation of mycelium, mixtures of algal and fungal growths in slimes on stones or other substrata, and techniques of sample collection, tend to disguise the nature of the total sample and the observer tends to ignore the presence of this segment of the population. In addition, the average observer of plankton and benthos samples, untrained in esoteric aquatic mycology, will have little or no basis for recognizing a fungus under his microscope.

B. Lytle Creek*

Geography

Lytle Creek lies in the Little Miami River Basin. It flows into Todd's Fork, a tributary of the Little Miami River. Its mouth is about 45 miles northeast of Cincinnati. It drains about 27 square miles of land about a third of which lies in the city of Wilmington, a community of 7,412 people, according to the 1950 census (1960: 8,915). Although Lytle Creek is now considered a permanent stream, the effluent from the Wilmington sewage treatment plant comprises 80-100% of the stream flow during the low flow stage in summer and autumn. The creek is approximately 11 miles long and has an average gradient of 20 feet per mile,

*Reproduced with permission from Ecology 42:1-18. 1961. Preliminary notes on this subject were published in Sewage and Industrial Wastes 26:790-794. 1954.

according to the Blanchester Quadrangle U.S.G.S. map (1931). It is 3-35 feet wide during low water stages and varies in depth from a few inches in the riffles to more than 6 feet in at least two pools.

East and north of the city its flow is weak, being strengthened by minor tributaries, outfalls and seepages from septic tanks and other run-off from farms in the region. Through the city it receives outfall from individual sanitary drains not tapped into the Wilmington sewage system, as well as street drainage and storm run-off. Just outside the western city limits it receives the outfall from the Wilmington sewage treatment plant. At various times the stream has received raw sewage, first because of a break in the trunk sewer about a half mile above the plant, and second because effluents from a dairy and a metal shop drain directly into the creek. At the time of samplings reported here, the sewage treatment plant was a primary-type with chemical precipitation and the runoff in the outfall was rich in organic matter. An average of approximately $3/4$ million gallons of sewage was treated daily. The sewage was of the combined type and during periods of heavy rain a large part of the total flow was bypassed directly into the creek. This resulted in a pool at the outfall whose bottom was highly septic. In the summer of 1954 the treatment process was changed to activated sludge which resulted in a marked change in the nature of the stream as mentioned in the chapter on aquatic fungi.

At the outfall from the sewage treatment plant a turbid condition occurred, although water generally appeared relatively clear. However, the growth on the stream bed and banks and the resulting odors indicated that, during the first mile, or at some seasons two miles below the

outfall, the turbidity supported a population of organisms related more to pollution than to clean water.

Habitat, Chemical, and Physical Characteristics

The stations on the creek (Fig. 1) were numbered according to their measured mileage from the mouth at Todd's Fork. They were chosen during the early phase of the biological study for their access to roads as well as for their abilities to demonstrate the typical biotic characteristics of the major pollution zones; thus they are not equidistant.

The major habitat conditions as of July 10, 1952, are recorded in Table 10. They are not necessarily those of the present time, as the creek bed at some places has been arbitrarily altered. Certain stream bed characteristics also have changed in the intervening years as a result of the change in sewage treatment which has resulted in a different type of effluent and aquatic environment.

The temperatures of the sampled habitats were taken in four sampling periods (Table 11). They were taken with a stab thermometer registering degrees centigrade, placed at the sampling points and allowed to come to equilibrium with the habitat before being read.

At each of the 8 stations, 4 types of samplings were taken by scooping materials to be tested into metal soil cans. 1. water from the stream, 2. water and sediment from a pool, 3. water and sediment from a riffle, and 4. bank soil which was always wet and always subject to extra irrigation during periods of high water. From a 1:10 dilution

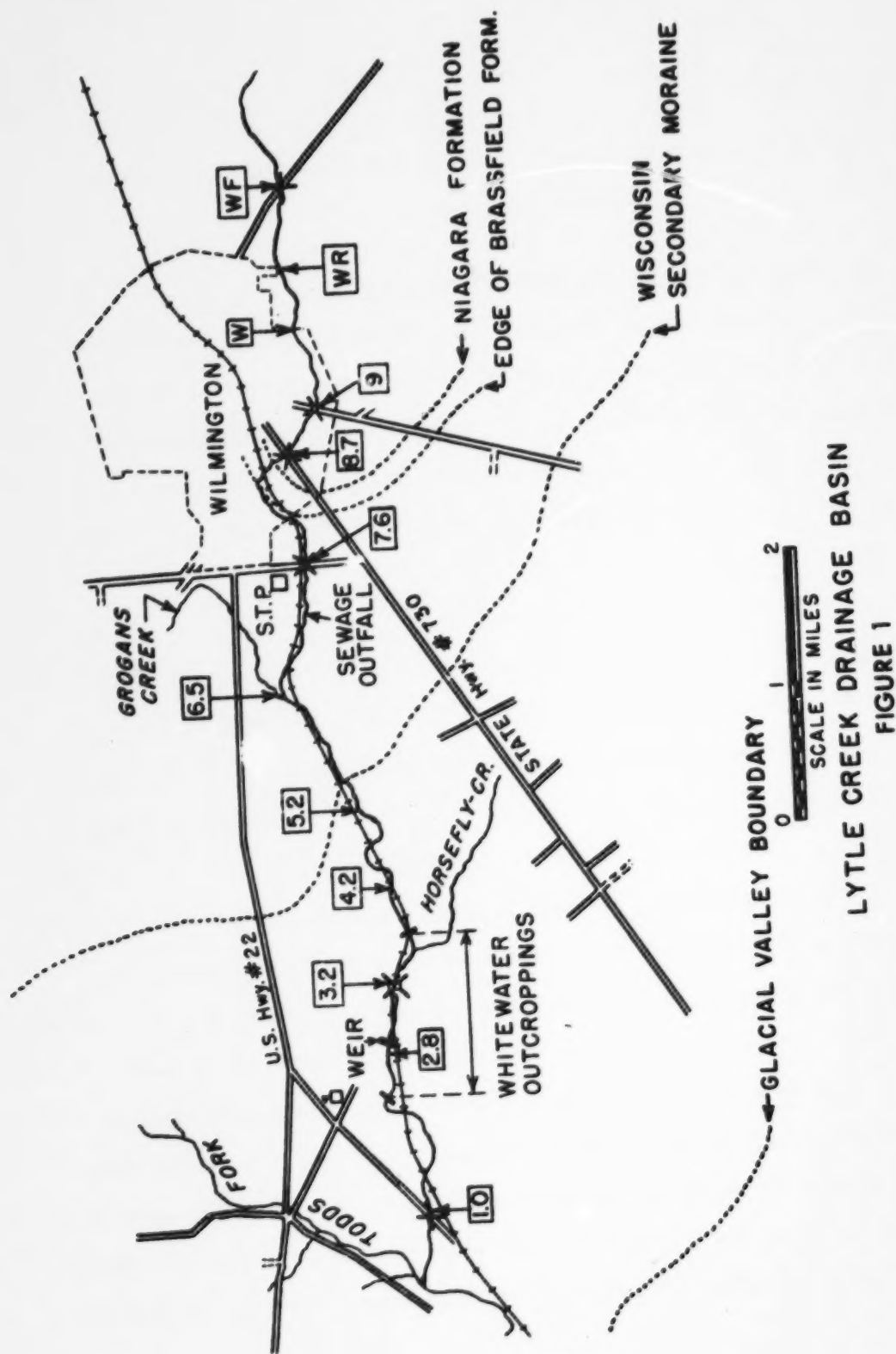


Table 10. Habitat Conditions, Lytle Creek, Clinton Co., Ohio. July 10, 1952

Station	Bed	Bottom	Bank	Border
1.0	Gravelly	Clean	Course sandy	Willow shrub
2.8	Flat native rock		Rock ledges	Grassy meadow with marshy springs.
4.2	Gravelly	Clean	Muddy to sandy	Tree lined
5.2	Gravelly	Masses of attached <u>Sphaerotilus</u>	Sandy to gravelly	Meadow with occasional sycamores
6.5	Gravelly	As 5.2 but with occasional blanket of <u>Sphaerotilus</u>	Sandy	Open meadow
7.2	Sandy-gravelly	Septic sludge	Muddy to sandy with septic organic matter	Tree and shrub lined: sewage treatment plant outfall.
7.6	Sandy-gravelly	Clean, green algae in pool	Sandy-gravelly	Tree lined; one bank formed from city dump refuse.
8.7	Gravelly	Clean	Sandy-gravelly	Willows and meadow, open.

Table 11. Temperatures of Lytle Creek habitats (in degrees C.).

Stations	9-18-52	<u>Dates</u>		
		1-14-53	2-25-53	3-30-53
		Water		
8.7	18.0	4.5	3.0	8.0
7.6	18.0	4.5	3.0	8.0
Effluent		9.0	7.5	11.0
7.2	21.5	6.0	5.0	9.5
6.5	19.0	6.5	5.0	9.5
5.2	19.0	5.0	4.0	8.5
4.2	18.5	4.5	3.5	7.5
2.8	18.5	4.0	2.5	8.0
1.0	18.5	3.5	2.5	8.0
		Water and sediment in pool		
8.7	18.0	5.5	3.0	7.0
7.6	18.0	5.0	3.5	7.0
7.2		7.0	4.0	11.5
6.5	19.5	7.0	4.5	8.5
5.2	19.0	5.5	4.0	8.0
4.2	18.5	4.5	4.0	7.0
2.8	18.5	4.0	4.5	8.5
1.0	18.5	8.0	6.0	9.0
		Water and sediment in riffle		
8.7	18.0	5.0	2.5	7.5
7.6	18.0	4.5	3.0	7.0
7.2		6.0	5.0	9.0
6.5	19.0	6.0	4.5	9.0
5.2	18.5	5.0	3.0	7.0
4.2	18.0	4.5	3.0	7.0
2.8	18.0	4.5	2.0	8.0
1.0	18.0	4.0	3.0	6.0
		Bank Soil		
8.7	18.0	6.0	1.5	8.0
7.6	18.0	5.0	1.5	8.0
7.2		5.0	3.0	16.5
6.5	18.0	5.5	3.5	9.0
5.2	18.0	5.0	1.0	7.0
4.2	17.5	4.5	0.5	9.0
2.8	17.5	4.5	1.0	12.0
1.0	17.0	5.0	2.0	8.5

Air temperature: 20-25

10.0-11.5

of each of these samples the supernatant was withdrawn and the pH was taken using a glass electrode pH meter (Table 12).

Data for temperature and precipitation at Wilmington, Clinton County, Ohio (Table 13) were accumulated from the records of the U. S. Weather Bureau and were converted to degrees Centigrade and millimeters of precipitation. In addition to maximum and minimum temperatures recorded at Wilmington for the dates on which samples were taken along Lytle Creek, this table presents the average maximum and minimum temperature for each month during the period of sampling. Only total monthly precipitation data are included. Without the sewage treatment plant effluent, the character of Lytle Creek would have been one of intermittent flow and varying levels. During the sampling period, this intermittent character was expressed more in flood conditions carrying excess runoff from heavy precipitation.

The temperature records clearly indicate that the temperature of the water in Lytle Creek is directly influenced by the temperature of the discharge from the sewage treatment plant regardless of the season. The water temperature is always warmer than the minimum air temperature and cooler than the maximum. For at least two miles below the Wilmington outfall, the temperature of the stream bottom in pools and riffles was also affected by the temperature of the sewage during the period in which temperatures were taken, except in February when the effect was noted for only a mile. Temperatures of the bank soil were so variable during the late winter and early spring that no correlation can be made between effect of sewage temperature and temperature of the creek water.

Table 12. pH levels of Lytle Creek habitats

Stations	Dates			
	9-18-52	1-14-53	2-25-53	3-30-53
			Water	
8.7	7.8	7.1	8.2	7.2
7.6	7.6	7.4	8.1	7.9
Effluent		7.3	8.0	7.6
7.2	7.4	7.3	8.1	7.8
6.5	7.6	7.2	8.0	7.8
5.2	7.8	7.3	8.4	7.8
4.2	7.9	7.2	8.2	8.0
2.8	7.8	7.4	8.1	8.2
1.0	7.8	6.6	8.2	8.0
		Water and sediment in pool		
8.7	7.9	7.8	7.3	7.2
7.6	7.6	7.8	7.0	6.9
7.2		7.4	7.2	7.1
6.5	7.1	7.3	6.8	7.0
5.2	7.5	7.5	7.4	8.0
4.2	7.6	7.5	7.2	7.2
2.8	7.4	7.6	7.8	7.2
1.0	7.5	7.7	7.5	7.2
		Water and sediment in riffle		
8.7	7.8	7.8	7.9	7.8
7.6	7.5	7.9	7.4	7.7
7.2		7.4	6.7	6.6
6.5	8.2	7.8	7.7	7.5
5.2	7.5	7.3	8.5	7.6
4.2	7.5	7.2	7.8	8.2
2.8	7.4	7.5	7.5	7.1
1.0	7.8	7.7	7.8	7.6
		Bank Soil		
8.7	7.6	7.8	7.7	7.3
7.6	7.6	8.2	7.5	6.4
7.2		7.8	7.3	8.3
6.5	7.9	7.1	8.3	7.7
5.2	7.6	7.6	7.6	7.4
4.2	8.0	7.8	8.5	8.2
2.8	7.9	7.7	8.3	7.0
1.0	8.2	7.5	7.4	8.2

Table 13. Temperature and precipitation data: Wilmington, Clinton Co., Ohio

Date	Temperature - degrees C		Precipitation		
	Monthly Average		mm. (Month)		
	Minimum	Maximum	Minimum	Maximum	
March 27, 1952	-0.83	10.0	-1.67	7.22	115.57
April 17	4.22	17.11	0.00	18.89	113.28
May 15	9.94	22.33	15.56	25.00	107.19
June 12	16.72	30.67	12.78	23.89	109.98
July 10	17.06	31.94	8.32	27.22	32.77
Aug. 17	14.89	29.72	16.11	30.56	66.29
Sept. 18	10.22	26.33	13.89	24.44	64.52
Oct. 23	1.61	17.44	-0.56	20.00	31.24
Nov. 26	0.56	12.22	3.89	14.44	49.28
Dec....	-2.28	5.28	----	----	108.71
Jan. 14, 1953	-3.06	6.67	2.78	12.78	145.29
Feb. 25	-3.78	8.39	-3.33	5.56	36.32
Mar. 30	0.28	11.78	-4.44	9.44	67.56

Other factors of the habitat which have not been considered in this study are probably operative here.

A number of facets of the biology of the several sampling stations on Lytle Creek were summarized by Cooke (1961a).

The Geofungi of Lytle Creek

To determine the relationship of fungi to the degree of pollution in a stream which receives effluent from a primary-type domestic sewage treatment plant, several techniques can be employed. One method permits the study of fungi associated with zoo- and phytoplankton as parasites of the living cells; another permits the isolation from the habitat of true aquatic fungi by means of hemp seed and other types of baits; another, the direct microscopic examination of the habitat to determine whether or not fungi are present; another permits determination from samples of the substratum of the amount of biological activity present by the use of various physiological techniques; and, finally, samples of the habitat may be treated as if they were soil samples, isolating from them by use of suitable media such fungi as might grow well on this medium. In counting the different types of organisms in the plankton, no report was made of parasitic chytrids. Three sets of samples were baited with hemp seed in the laboratory with no especially significant results. In direct microscopic examination of benthos samples (Table 14) a record was made in terms of "bacteria and fungi - microscopic count, millions per square foot". From the comments accompanying the data it would appear that "fungi" in this instance referred to "sewage fungus",

Table 14. Data on bottom deposits in Lytle Creek, Week of Dec. 6-13, 1949

Category	7.6	7.2	6.5	Stations 5.2	4.2	2.8	1.0
Water Temperature °F				Riffles			
Total Nitrogen as N ₂ -P.P.M.*	32	42.8	41	35	32	34	51
Total Phosphorus as PO ₄ -ppm*	30	800	150	70	58	330	90
Volatile solids over total solids - %	107	206	50	76.7	132	40.2	34.7
Coliform M.P.N./sq. ft.	39.2	49.6	23.7	32.3	36.1	24.1	6.87
Bacterial growth 20 C - 48 hr. Billions M.P.N./sq. ft.	445M**	1367B	55.5B	55.2B	2.5B	257M	892M
Bacteria and Fungi - microscopic count, Millions/sq. ft.	765.2	13750.0	295	899	263	654	273M
Blue-green algae, Millions/sq. ft.	103.8	1476.9	1476.9	315.6	35.6	4.23	0.63
Diatoms, Millions/sq. ft.	3.75	4.66	4.66	13.18	152.17	8.45	1.26
Flagellates, Millions/sq. ft.	611.9	2062.9	2062.9	39.6	1135.04	351.82	69.93
Rhizopods and Ciliates, Millions/sq. ft.	11.25	5.83	5.83	5.64	54.7	107.0	17.62
	2.5			1.88	1.37	1.41	
Water Temperature °F				Pools			
Total Nitrogen as N ₂ -P.P.M.*	32	42.8	41	35	32	34	51
Total Phosphorus as PO ₄ -ppm*	400	600	440	420			
Volatile solids over total solids - %	52.3	364	306	86.5			
Coliform M.P.N./sq. ft.	9.05	47.9	43.4	10.8			
Bacterial growth 20 C - 48 hr. Billions MPN/sq. ft.	.25M	1.02B	248M	46.5M	10.5M	2.48M	4.65M
Bacteria and Fungi - microscopic count, Millions/sq. ft.	120M	1.2B	4.65B	4.65B	248M	259M	465M
Blue-green algae, Millions/sq. ft.	19.6	4802.16	4802.16	49.78	4.5	2.03	
Diatoms, Millions/sq. ft.	1.82	348	348	97	5.41		
Flagellates, Millions/sq. ft.	17.85	13.34	13.34	10.47	4.96	112.03	106.81
Rhizopods and Ciliates, Millions/sq. ft.	.46	109.3	109.3	159.25	8.56	95.2	71.2
		8.93	8.93	8.89		22.4	.46

* Wet basis

** M - Million; B - Billion

Based on cells and cell clusters

Note: Use of word "fungi" refers to *Sphaerotilus natans*

Sphaerotilus natans, a filamentous bacterium. A purely physiological study of the activities of organisms in the benthos or plankton was not considered practical until something was known of the members of the population. Such a study would have involved the activities of many kinds of organisms in addition to those of the fungi. It was hoped that the plating of habitat samples from each major habitat type in each zone, which had been analyzed for other kinds of organisms, would yield information concerning the kinds and numbers of fungi occurring in the creek under various levels of change resulting from pollution.

Our concern in reporting on Lytle Creek fungi is to indicate that geofungi are present in the benthos and plankton of a stream, whether they be found in the water itself, in sediments in pools and riffles, or even in the soil of the creek bank. In addition to listing the species which have been found, together with information about their occurrence in the various habitats of a stream (Table 15), their occurrence is summarized graphically in the accompanying histograms (Figs. 2 and 3).

In Table 16 are listed most of the species of geofungi collected during the 12-month study period in 1952 and 1953. The list includes 94 categories, among them certain collectives. In addition, members of 34 categories have been found only once in any one location and are not listed here. The collectives include groups of cultures which have not been completely identified as yet. Thus the category "white yeasts" includes species in the genera Candida, Cryptococcus, Torulopsis,

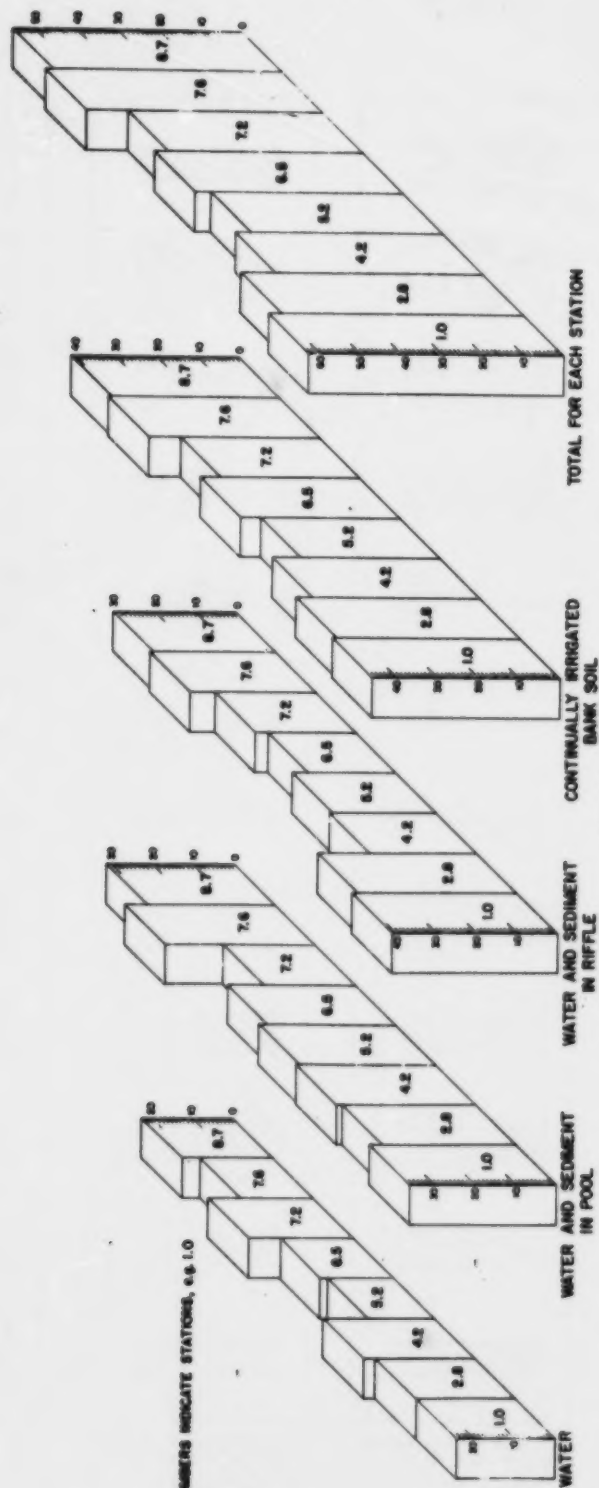
Table 15. Occurrence of Fungi at Sampling Stations, Little Creek.
(W - creek water; P - water and sediment in pools; R - water and sediment in riffles; S - bank soil.)

Fungus	1.0	2.8	4.2	5.2	6.5	7.2	7.6	8.7	Sampling Periods (of 12)
<i>Aspergillus versicolor</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Fusarium aqueductum</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	11
<i>Phialophora jeanselmei</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	12
<i>Penicillium funiculosus</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	12
<i>Penicillium lilacinum</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	12
<i>Penicillium ochrochloron</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	11
<i>Penicillium variable</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Aureobasidium pullulans</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Endothorax putinis</i> sp. lat.	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
White yeasts									
<i>Peziza</i> spp.	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Geotrichum candidum</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	12
<i>Cladosporium cladosporioides</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Trichoderma viride</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	12
<i>Penicillium</i> spp.	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	12
<i>Moniliaceae</i> spp.	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Penicillium chrysogenum</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Coniothyrium</i> spp.	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	3
<i>Penicillium nigricans</i> (A nr.)	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Fusarium oxysporum</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	7
<i>Penicillium rugulosum</i>	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	3
<i>Aspergillus fumigatus</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	12
<i>Alternaria tenuis</i> sp. lat.	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Aspergillus ochraceus</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Gliocladium roseum</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Xylotheclium variegatum</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	10
<i>Penicillium herquei</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	8
<i>Penicillium oxalicum</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	11
<i>Penicillium fallaxianum</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	5
<i>Aspergillus clavatus</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	9
<i>Acrocarpon frigidum</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	11
<i>Penicillium lanthornellum</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	9
<i>Penicillium purpureogenum</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	8
<i>Acrocarpon plumbeum</i>	PRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	WPRS	9

80m

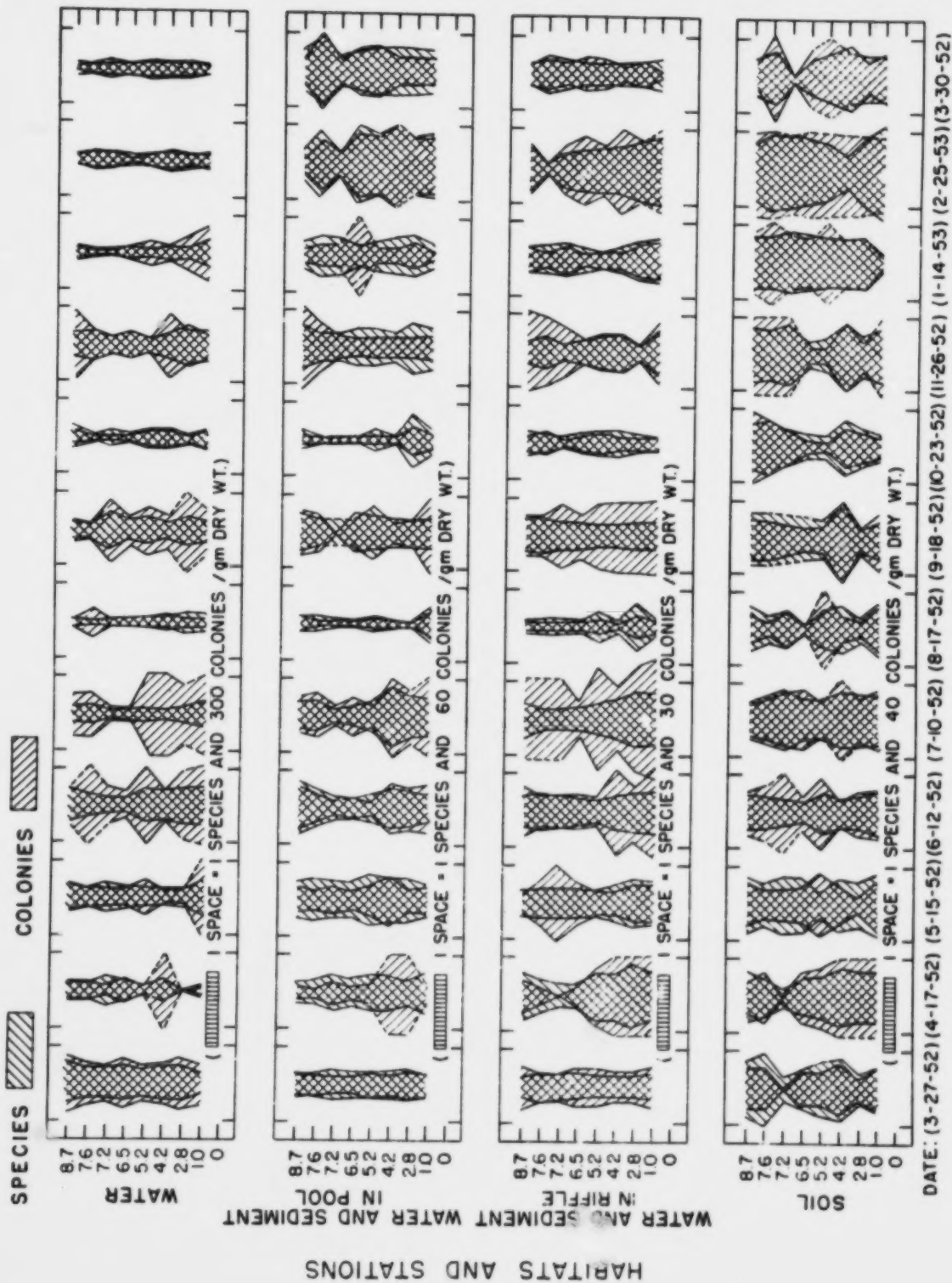
Table 15. p. 2

<u>Aspergillus niger</u>	WRS	PS	WPRS	WS	RS	PS	12
<u>Penicillium puberulum</u>	WPR	PS		P	RS	P	2
<u>Aspergillus nidulans</u>	WP S	PR		WR	PS	WPRS	4
<u>Penicillium martensii</u>	S	P	S	WP	PS		3
<u>Penicillium velutinum</u>	S	S	PS	R	S	P	1
<u>Coniothyrium fuckelii</u>	S	PS	PS	WS	RS	PS	3
<u>Mucor bisseialis</u>	S	PS	PS	S	S		3
<u>Mucor racemosus</u>	WP S	S	PS	W	W		3
<u>Fusarium solani</u>	WR			RS	RS	PRS	3
<u>Aspergillus clavatus</u>	PRS		P		PRS	PRS	7
<u>Penicillium expansum</u>	PR	RS		RS	PS	RS	3
<u>Penicillium variotii</u>	PS		R		P	WPR	3
<u>Aspergillus terreus</u>	RS	PS	RS		S	S	6
<u>Penicillium lanosum</u>	RS	PS		RC	RS	RS	7
<u>Penicillium charlesii</u> († nr.)	S	WS	P	S	S	S	3
<u>Rhizopus arrhizus</u>		S	P		P	S	5
<u>Aspergillus flavus</u>	PR		S	WS	W	S	5
<u>Penicillium javanicum</u> († Ser.)	PS		S	S	P	W	2
<u>Epicoecum purpurascens</u>	R	WP		S	W	R	3
<u>Fusarium roseum</u>	R	PS	W		PS		2
<u>Doratomyces stemonitis</u>	S	PS	R	R	P	PS	1
<u>Zygorynchus moelleri</u>	S	PS	S		S	PS	3
<u>Verticillium sp.</u>		P	S		W	W	3
<u>Thielavia sp.</u>					R		1
<u>Aspergillus ustus</u>	WPR	S				PR	4
<u>Chaetomium funiculosum</u>	P						2
<u>Penicillium commune</u>	RS	S					3
<u>Penicillium tardum</u>	R	WR					2
<u>Sphaeronema spp.</u>		RS				S	2
<u>Penicillium brevicompactum</u>	WP	S	R	W			2
<u>Penicillium casei</u> († nr.)	W	PRS	R	W			2
<u>Gliocladium penicillatum</u>	P	P			P		3
<u>Pestalotia dictyota</u>	P					PR	1
<u>Fusidium viride</u>	R			R	R		1
<u>Monosporium olivaceum</u>	R					S	1
<u>Penicillium cyano-fulvum</u>	S		P		W		2
<u>Penicillium raistrickii</u>	S	R					2
<u>Mucor brunneus</u>		S	S				1
<u>Curvularia lunata</u>	RS				S		2



PERCENT OF TOTAL (127) SPECIES IN EACH SAMPLING SITE

FIGURE 2



Trichosporon, and Schwanniomyces. The category of "Phoma spp." includes what appear to be several species in addition to P. herbarum and P. terrestris. There is a number of cultures which have not been identified beyond the families Moniliaceae and Dematiaceae because either they have never sporulated or their spore-bearing mechanisms have not yet been observed accurately enough to complete an identification. Certain strains of Penicillium have not yet been identified to species or were discarded before identification was completed. In this table the categories are listed according to their occurrence in the stream. Thirty-five categories were found at least once in all 8 stations, while only 10 categories were found in all habitats sampled in all 8 stations. The last column in Table 16 indicates the number of times of the 12 sampling periods that the category listed was found at any point in the stream.

We may arbitrarily assign these fungi to one of two of the categories adopted earlier for pollution fungi (see Chapter on Indicator Organisms, p.302). None of the fungi found is lymabiontic, restricted to a grossly polluted habitat. Those found in 5 or more sampling periods, according to Table 16, may be considered lymaphilic: species able to tolerate a pollution habitat as a liveable one. Those species not tolerating such a habitat but merely passing through, so to speak, are lyma-xenic; they are the species found in fewer than 5 sampling periods. Since lymaphobes cannot tolerate conditions of pollution, none was found in Lytle Creek, although some of the species found only once or twice, as a result of capturing a chance spore, may have been lymaphobes. This analysis ignores the fact that a few species or categories were found only once

Table 16. Fungi observed in isolations from Lytle Creek, Clinton Co.,
Ohio

Species	Observation Number	Number of Dates (of 15)
<u>Penicillium lilacinum</u>	308	12
<u>Trichoderma viride</u>	274	14
<u>Penicillium funiculosum</u>	266	13
<u>Penicillium</u> spp.	249	14
<u>Geotrichum candidum</u>	197	14
<u>Penicillium ochrochloron</u>	191	13
<u>Aureobasidium pullulans</u>	183	11
<u>Phialophora jeanselmei</u>	180	13
<u>Fusarium aquaeductum</u>	155	12
<u>Aspergillus versicolor</u>	151	10
<u>Aspergillus fumigatus</u>	142	13
<u>Moniliaceae</u>	141	13
<u>White Yeasts</u>	131	11
<u>Cladosporium cladosporioides</u>	125	13
<u>Rhodotorula</u> spp.	108	11
<u>Penicillium variabile</u>	107	11
<u>Phoma</u> spp.	102	12
<u>Mucor fragilis</u>	96	13
<u>Pythium</u> spp.	79	2
<u>Dematiaceae</u>	78	10
<u>Penicillium oxalicum</u>	68	12
<u>Gliocladium roseum</u>	67	10
<u>Alternaria tenuis</u>	57	12
<u>Aspergillus niger</u>	50	13
<u>Coniothyrium</u> spp.	49	8
<u>Mucor</u> spp.	47	6
<u>Aspergillus ochraceus</u>	45	10
<u>Myrothecium verrucaria</u>	44	12
<u>Penicillium purpureogenum</u>	42	11
<u>Penicillium chrysogenum</u>	41	5
<u>Penicillium janthinellum</u>	41	10
<u>Penicillium herquei</u>	29	8
<u>Penicillium nigricans</u>	28	7
<u>Fusarium oxysporum</u>	26	6
<u>Aspergillus clavatus</u>	22	8
<u>Fusarium solani</u>	21	6
<u>Penicillium lanosum</u>	20	8
<u>Aspergillus terreus</u> var. <u>terreus</u>	19	6
<u>Aspergillus flavipes</u>	18	10
<u>Aspergillus sydowii</u>	16	4
<u>Mucor plumbeus</u>	16	6
<u>Fusarium</u> spp.	15	4
<u>Penicillium expansum</u>	15	5
<u>Penicillium puberulum</u>	15	3
<u>Saprolegnia</u> spp.	13	3
<u>Phoma herbarum</u>	11	3
<u>Aspergillus flavus</u>	10	7
<u>Penicillium javanicum</u> Ser.	10	3
<u>Paecilomyces varioti</u>	9	7
<u>Rhizopus arrhizus</u>	9	6
<u>Mucor racemosus</u>	8	4
<u>Saprolegnia delica</u>	8	2
<u>Aspergillus</u> spp.	7	3
<u>Aspergillus ustus</u> var. <u>ustus</u>	7	4
<u>Fusarium roseum</u>	7	3

<u>Mucor hiemalis</u>	7	4
<u>Paecilomyces</u> spp.	7	3
<u>Verticillium</u> spp.	7	2
<u>Coniothyrium fuckelii</u>	6	3
<u>Penicillium brevicompactum</u>	6	2
<u>Saprolegnia ferax</u>	6	2
<u>Zygorhynchus moelleri</u>	6	6
<u>Achlya americana</u>	5	2
<u>Doratomyces stemonitis</u>	5	4
<u>Epicoccum purpurascens</u>	5	4
<u>Memnoniella echinata</u>	5	3
<u>Penicillium commune</u>	5	4
<u>Penicillium martensii</u>	5	5
<u>Penicillium velutinum</u>	5	2
<u>Chaetomium funiculosum</u>	4	3
<u>Dictyuchus monosporus</u>	4	4
<u>Gliomastix convoluta</u>	4	3
<u>Monosporium apiospermum</u>	4	3
<u>Penicillium charlesii</u>	4	1
<u>Penicillium parvum</u>	4	3
<u>Penicillium raistrickii</u>	4	2
<u>Sphaeronema spinella</u>	4	4
<u>Thielavia</u> sp.	4	2
<u>Verticillium lateritium</u>	4	2
<u>Aspergillus ustus</u> var. <u>laevis</u>	3	1
<u>Curvularia</u> sp.	3	2
<u>Fusidium</u> sp.	3	1
<u>Mucor corticolus</u>	3	1
<u>Paecilomyces elegans</u>	3	1
<u>Penicillium casei</u>	3	1
<u>Penicillium cyaneofulvum</u>	3	2
<u>Penicillium jensenii</u>	3	1
<u>Penicillium pulvillorum</u>	3	2
<u>Penicillium rugulosum</u>	3	1
<u>Pestalotia dichchaeta</u>	3	1
<u>Preussia dispersa</u>	3	2
<u>Stilbellaceae</u>	3	2
<u>Trichoderma alba</u>	3	2
<u>Aspergillus candidus</u>	2	2
<u>Aspergillus carneus</u>	2	2
<u>Aspergillus ruber</u>	2	2
<u>Aspergillus wentii</u>	2	1
<u>Fusarium chlamydosporum</u>	2	2
<u>Gliocladium</u> sp.	2	2
<u>Gliocladium catenulatum</u>	2	2
<u>Paecilomyces marquandii</u>	2	2
<u>Penicillium aurantiovirens</u>	2	1
<u>Penicillium citreoviride</u>	2	1
<u>Penicillium palitans</u>	2	1
<u>Syncephalastrum racemosum</u>	2	2
<u>Pestalotia versicolor</u>	2	2

The following species were observed only once in this series of samples:

Absidia ramosa, Aspergillus terreus var. floccosus, Cephalosporium spp.,
Chaetomium sp., Cladosporium herbarum, Cytospora sp., Fusarium tricinctum,
Geolegnia inflatum, Oliocladium penicillatum, Monilia sitophila, Monosporium
olivaceum var. major, Mucor brunneus, M. nr. jensenii, Penicillium citrinum f.,
P. claviforme, P. daleae, P. nr. ehrlichii, P. fellutanum, P. implicatum,
P. kapuscinskii, P. nr. nalgovensis, P. notatum, P. stipitatum, P. thomii,
P. vermiculatum, Phialophora sp., Phoma terrestris, Phycomycete sp.,
Polyporus sp., Pseudogymnascus roseus, Pyrenochaeta sp., Saccharomyces
sp., Scopulariopsis chartarum, Sphaeronema sp., Sporotrichum epigaeum var.
terrestre, Sp pruinatum, Stachybotrys atra, tan yeast, yellow yeast,
Zygorhynchus heterogamus.

and that some species were found more than once but in a portion of the stream still influenced to a small extent by upstream pollution. It is further assumed that those categories occurring 4 or fewer times have not become permanent members of the population, while those found 5 or more times have developed a continuity of existence within the series of environments which make up the sampling limits of the stream.

Figure 2 summarizes these data as a series of histograms based on the percentage of the total number of species categories present in any one of the 32 sampled habitats. The first column represents those species found in water samples taken at the 8 stations, the second, those found in water and sediment in pools, the third, water and sediment in riffles, and the fourth represents those categories found in saturated bank soil always subject to the irrigative influences of the stream water and always flooded during periods of high water. The final column summarizes these data for each station.

For each station and each habitat the environment is in a continual state of flux. The stream is sufficiently small that the water is continually moving downstream and there is little chance for long periods of retention in backwater pools. During the late summer and autumn, and during periods of winter ice, water movement may be slow, but during rainy periods the pools and riffles are continually scoured, although this action may be too mild to affect many of the animals which are present in an attached state. To a certain extent these features of water movement are reflected in the histograms in Fig. 2.

Each set of histograms shows slightly different relationships between the species of fungi and their habitats. The water in the upper

part of the stream carries a quantity of spores which is reduced as the stream passes the break in the trunk sewer line but which increases as the full load of the effluent of the sewage treatment plant is emptied into it. This variation is followed by a decline in numbers of species as the septic zone is passed. As oxygen supplies are increased, pollution loadings decreased and the full effects of enrichment are felt, the load of species is again increased.

It is apparent that an increase in nutrients entering the water and sediment in pools causes an effect on the fungus population. As the enrichment effects of the raw sewage from the trunk line sewer break are felt, populations increase, but at the point of outfall the number of species sharply declines. This condition is attributed to the highly septic conditions which affect only certain species, as colony counts demonstrate. Proceeding downstream, then, there is a gradual increase in the numbers of organisms which can adapt to waters carrying an increasingly mild load of fertilizing materials. At Station 2.8 the stream flows over flat limestone rocks providing little chance for development of sediments of the type sampled elsewhere.

The pattern in the riffles is similar to that in the pools except that the most severe influence of pollution is felt at Station 6.5 rather than at 7.2. Here there is a more rapid increase in the number of species present after the zone of recovery is almost passed.

In the bank soil there is a good population of many species throughout the stream although this is depressed at two points. At Station 7.2 the depressing effect is explained on the basis of sewage

outfall and accompanying septicity which is obvious as a deep soft layer of black mud. Some recovery is noted at Station 6.5 which is lost again at Station 5.2, beyond which recovery is noted along the length of the stream. The depression at Station 5.2 may be explained partially on the basis of habitat variation, although nothing in the habitat conditions appeared to account for this beyond the unaesthetic appearance and odor of the stream. It was a point in a stream in which a careful parent would not permit his children to play, to describe the condition qualitatively.

The depressing effect on the population of fungal species at Station 5.2 is again noted in the summary histograms which follow the pattern set up by the bank soil populations. Here is noted the effect of the raw sewage entering at a point above Station 7.6 as well as the fertilizing effect on downstream populations of the sewage pollution received at Station 7.2.

In Fig. 2 no attempt is made to differentiate between seasons, the data presented being based only on the total number of species found in the stream. Fig. 3 presents information on a month-to-month basis for each of the 4 habitats at each of the 8 stations, comparing colony counts with numbers of species for each of the 32 habitats sampled. Here December is omitted but March is repeated; otherwise the pattern for a complete year is presented. Sampling stations are shown on the left of each of the 4 habitat types; sampling dates are given at the bottom of each of the 12 columns. Cross-hatching for numbers of colonies runs from upper left to lower right, usually the inner pair of enclosing lines includes the numbers of species for which one space on the scale

indicates one species. The outer pair of enclosing lines usually includes the numbers of colonies for which one space represents 300 colonies per gram dry weight of water, 60 colonies per gram dry weight of water and sediment in pools, 30 colonies per gram dry weight of water and sediment in riffles, and 40 colonies per gram dry weight of water-soaked bank soil.

This set of histograms illustrates a very definite reduction in numbers of species and colonies as a result of the added pollution in the stream at or just below the point of discharge of primary settled sewage. This effect is noted at Station 7.2 or 6.5 depending on the rate of flow of the stream water.

A quiet period in the life of the stream itself is demonstrated by relatively regular lines representing both species and colonies. Unusual disturbance, such as special types of polluttional loadings, temperature conditions, precipitation conditions, or other causes result in moderate to strong variations in the shape of the curves enclosing the data for the sampling period involved.

Discussion

Many kinds of fungi have become adapted to habitats such as may be found in streams. Natural streams have been subject to polluttional influences for as long as organisms have lived along their reaches, a situation considered objectionable only during the last 50-100 years. It is suggested that soil fungi became adapted to the aquatic environment through their natural ability to break down organic polluttional matter. However, the postulation of such a thesis may be unnecessary

since it is probable that soil fungi, finding their way into streams or sewage treatment plants, through runoff from the soil, washings resulting from flash floods, precipitation of spores from the air, and other ways, have become adapted to these habitats by merely being bathed in an adequate supply of nutrients, which they incidentally aid in reducing to the mineral level.

Within the confines of the particular stream, Lytle Creek, between 8.7 and 1.0 miles above its mouth and about 3 miles from its source, a large and varied population of soil fungi has been found. Based on these soil molds it is suggested that a large population representing strains of at least 35 species has been found to occur at most of the sampling stations throughout most of the year, and that 10 of these have been found at all stations in 10, 11, and 12 of the 12 sampling periods.

The populations of soil fungi found in Lytle Creek add another example to the growing list of habitats to which fungi are known to be adapted. Here, in a completely aquatic habitat, these fungi are found, by means of pure culture techniques, to have joined the ranks of those scavenger organisms referred to by Whittaker (1957) as "reducers" and appear to be aiding in stream purification.

The occurrence of such fungi in aquatic environments could have been predicted since they have developed for students of aquatic fungi as contaminants of cultures ever since such fungi have been studied. Rather than interpret these as merely culture contaminants, it is emphasized here that they are definite members of the population, that they

should be studied as such, and that their role in the habitat should be considered of fundamental importance in aquatic biology. Such fungi are capable of contributing to the reduction of B.O.D. in aquatic habitats, as has been demonstrated with 8 of the common species by Cooke, Moore and Kabler (1957) (see Chapter XII, Section D). The same group of fungi is capable of using many kinds of carbohydrates and nitrogen sources without the addition of vitamin supplies, as has been demonstrated by Cooke (1957) (see Chapter XII, B). While some of these organisms may become nuisances and even undesirable organisms in human disease (Cooke and Kabler, 1955) (see Chapter X, A), and in plant disease (Cooke and Kabler, 1957) (see Chapter X, B) it seems apparent that as long as they are confined to an aquatic habitat where airborne spores are produced at a minimum, they are contributing to stream purification by reduction of organic waste materials through metabolic activities. Thus, the same organism which may be detrimental to man's interest in one environment, may be highly beneficial in another environment.

The activities of microorganisms in any environment may be overemphasized by one technique of study; underemphasized by another. To look for an accurate description of a fungus population in a Sedgwick-Rafter cell or a Palmer Mammoplankton counting cell is to underemphasize that population, since few spores or thalli are readily apparent, while to rely solely on plate counts based exclusively on the techniques described above is to overemphasize this segment of the total biota. This is the import of a sentence in a recent evaluation of micro-techniques by G. S. Wilson (1959) paraphrased as follows: "There are many faults

and fallacies in microbiology such as overemphasis on the numbers rather than the nature of microorganisms in assessing the populations of microorganisms in any habitat."

Given the proper technique for sorting out the individual organism, the different groups of organisms, and the organisms with highly specialized requirements for nutrients or tolerances for microenvironments, each organism can be placed in its proper niche in the picture of the ecology of a stream. Until such techniques are available for geofungi, information can be developed in the laboratory concerning the role of each strain or group of strains in the environment. On this basis we may say that geofungi isolated from the water and streambed samples from Lytle Creek are contributing to the reduction of organic pollutants in that stream, and that they are probably contributing to the food supplies of larger organisms or of organisms higher in the food chain which may ingest them accidentally or intentionally.

C. The Bear River

Geography

The Bear River, one of the streams on the eastern edge of the Great Basin, originates in a group of creeks draining the north slopes of the Uintah Mountains, Summit County, Utah. It flows north into Uintah County, Wyoming, thence northwest into Bear Lake County, Idaho, where it accepts the waters of Bear Lake, a large natural lake at the foot of the east slopes of the northern end of the Wasatch Mountains. It reaches its northernmost point at Soda Springs, Caribou County, Idaho, where it

rounds the Bear River Range of the Wasatch Mountains, and flows south into the Cache Valley of Franklin County, Idaho, and Cache County, Utah, from which it escapes through an artificial impoundment, the Cutler Reservoir, formed by a dam in a natural gorge through a small range west of the Wasatch, into the Great Salt Lake Valley. It finally empties into the Great Salt Lake.

The Cache Valley in Idaho and Utah is intensively cultivated. Irrigation waters are obtained from storage reservoirs supplied by the Bear River and its tributaries on the western slopes of the Wasatch Mountains, the Cub River, Worm Creek, and other smaller streams. Joining the Bear River at the head of Cutler Reservoir is the Logan River, which originates on the western slopes of the Wasatch Mountains east of Logan, Cache County, Utah. Near the city of Logan, the Logan River receives the waters of the Little Bear River, which drains additional agricultural lands in the southern part of Cache County. The Logan-Little Bear River system lies almost entirely within Cache County.

The uses to which the water of the Bear River and its tributaries in the Cache Valley are put, as well as the effects of these uses on the water and its quality, are described in the report: "Survey of Interstate Pollution on the Bear River and its Tributaries, Utah-Idaho, 1962," (PHS, 1963). This report on the isolations of fungi from samples collected in June and November, 1962, supplements the section "Stream Biology" of that report.

Techniques

Samples were collected during each of three of the different surveys

conducted in the Cache Valley. The first sample studied was obtained at about Station 14 in the autumn of 1961. This was returned to Cincinnati in a small vial. In the bacteriological survey conducted in June, 1962, grab samples of soils and creek bank muds and sediments were obtained from 12 stations. All but one of the sample stations correspond to those from stations used in November of the same year. In November, 1962, grab samples were taken for a comprehensive survey of the Cache Valley stations. At the same time, DuWayne F. Goodwin (Professor of Range Management, Utah State University), collected grab samples of soils, river and creek bank sediments, and spring water from the Logan River valley. Some of the habitats sampled had been disturbed by man and some by livestock; others had no history of recent disturbances. Samples were sent to Cincinnati by air-mail or other means as soon as possible, and refrigerated overnight before isolation procedures were initiated. Isolations were effected by hemp seed baits for true water molds, pour plates for colony counts and mold colony types, and shaken flask cultures for yeasts.

Results

Through the use of these techniques, described in detail in Chapter II, 109 species or species-groups of fungi (Table 17a) were recovered from 32 sets of samples of Cache Valley soils and sewages, and 14 samples from the Logan River valley. The fungi are listed by constancy classes in Table 17b. The isolates from the 46 stations were grouped in the 10 habitat types described in Table 18 - five from each of the two river systems from which samples were obtained. The habitat types were based on the amount of apparent use the waters of the streams received during their flow through the affected valleys.

Table 1/a. Fungi observed in isolations from the Bear River,
Utah-Idaho

Species	Observation Number
<u>Penicillium spp.</u>	169
<u>Moniliaceae spp.</u>	119
<u>Geotrichum candidum</u>	82
<u>Mucor spp.</u>	81
<u>Trichoderma viride</u>	58
<u>Phialophora jeanselmei</u>	55
<u>Mucor alternans</u>	43
<u>White yeasts</u>	36
<u>Candida krusei</u>	34
<u>Dematiaceae</u>	33
<u>Cephalosporium spp.</u>	32
<u>Candida parapsilosis</u>	30
<u>Phoma spp.</u>	30
<u>Cladosporium cladosporioides</u>	29
<u>Fusarium aquaeductum</u>	28
<u>Aspergillus fumigatus</u>	27
<u>Aureobasidium pullulans</u>	24
<u>Penicillium lilacinum</u>	24
<u>Alternaria tenuis</u>	23
<u>Cryptococcus diffluens</u>	20
<u>Rhizopus spp.</u>	20
<u>Aspergillus niger</u>	19
<u>Candida tropicalis</u>	18
<u>Fusarium oxysporum</u>	17
<u>Rhodotorula mucilaginosa</u>	17
<u>Stachybotrys atra</u>	17
<u>Aspergillus ochraceus</u>	16
<u>Hansenula californica</u>	16
<u>Verticillium lateritium</u>	16
<u>Paecilomyces varioti</u>	15
<u>Stemphylium sp.</u>	13
<u>Cryptococcus laurentii</u>	12
<u>Aphanomyces sp.</u>	11
<u>Achlya sp.</u>	10
<u>Aspergillus ustus</u>	11
<u>Aspergillus flavus</u>	9
<u>Fusarium solani</u>	9
<u>Kloeckera apiculata</u>	9
<u>Gliocladium roseum</u>	8
<u>Stemphylium botryosum</u>	8
<u>Cladosporium sp.</u>	7
<u>Curvularia sp.</u>	7
<u>Mucor hiemalis</u> Sect.	7
<u>Rhodotorula texensis</u>	7
<u>Trichosporon cutaneum</u>	7
<u>Candida sp.</u>	6
<u>Fusarium spp.</u>	6
<u>Gliocladium spp.</u>	6
<u>Cladosporium avellaneum</u>	6
<u>Candida intermedia</u>	5
<u>Candida utilis</u>	5
<u>Epicoccum purpurascens</u>	5
<u>Myrothecium verrucaria</u>	5
<u>Rhizolpidium sp.</u>	5
<u>Rhodotorula glutinis</u>	5
<u>Torulopsis famata</u>	5

Table 17a. p. 2

<u>Torulopsis glabrata</u>	5
<u>Verticillium</u> sp.	5
<u>Aspergillus</u> spp.	4
<u>Aspergillus tamarii</u>	4
<u>Cryptococcus luteolus</u>	4
<u>Penicillium funiculosum</u>	4
<u>Absidia spinosa</u> var. <u>biappendiculata</u>	3
<u>Aspergillus flavipes</u>	3
<u>Aspergillus wentii</u>	3
<u>Fusarium culmorum</u>	3
<u>Penicillium chrysogenum</u>	3
<u>Penicillium variabile</u>	3
<u>Penicillium velutinum</u>	3
<u>Rhizopus arrhizus</u>	3
<u>Rhodotorula</u> spp.	3
<u>Stemphylium consortiale</u>	3
<u>Syncephalastrum racemosum</u>	3
<u>Torulopsis stellata</u>	3
<u>Trichosporon</u> sp.	3
<u>Actinomucor elegans</u>	2
<u>Allomyces</u> sp.	2
<u>Aspergillus versicolor</u>	2
<u>Candida pulcherrima</u>	2
<u>Candida robusta</u>	2
<u>Chaetomium globosum</u>	2
<u>Cryptococcus albidus</u>	2
<u>Dictyuchus</u> sp.	2
<u>Fusarium avenaceum</u>	2
<u>Fusarium monifforme</u> var. <u>subglutinans</u>	2
<u>Helminthosporium</u> sp.	2
<u>Memnoniella echinata</u>	2
<u>Penicillium commune</u>	2
<u>Penicillium corymbiferum</u>	2
<u>Penicillium ochrochloron</u>	2
<u>Scopulariopsis brevicaulis</u>	2

The following species were observed only once in this series of samples:

Absidia ramosa, Arthrobotrys oligospora, Ascomycete, Aspergillus nidulans var. latus, A. sydowii, A. terreus var. terreus, A. terreus var. floccosus, Botryotrichum sp., Candida guilliermondii, C. pelliculosa, C. pseudotropicalis, C. solani, C. ?zeylanoides, Chaetomium sp., Cladosporium herbarum, C. macrocarpum, Conidiobolus villosus, Coniothyrium sp., Cytospora sp., Doratomyces stemonitis, Fusarium acuminatum, Gliocladium catenulatum, Mucor petropolitanus Sect., Penicillium herquel, Penicillium nigricans, P. sclerotiorum, Phialophora sp., Pichia membranaefaciens, Rhizopus chinensis Group, R. stolonifer, Rhodotorula graminis, R. ?pennaeus, Saprolegnia sp., Scopulariopsis asperula, S. chartarum, Sepedonium sp., Torulopsis aerea, T. andida, T. holmii, T. inconspicua, Trichosporon margaritifera, Ulocladium tuberculatum.

Table 17b

List of fungus species arranged according to occurrence
in habitat types and constancy classes

Habitat types	A	O	B	V	S	AP	RB	FT	U	S
Constancy	3	10	2	12	5	3	3	3	4	1
Class	3	10	3	19	10	6	3	4	8	1
I										
Fungus										
<u>Moniliaceae spp.</u>	P°	P	P	P	P°	P°	P°	P	P°	P°
<u>Phialophora jeanselmei</u>	P	P	P	P	P	P	P	P	P	P
<u>White Yeast spp.</u>	P°	P°	P°	P°	P°	P°	P°	P	P°	P°
<u>Mucor spp.</u>	P°	P	P	P	P	P	P	P	P	P
<u>Aspergillus fumigatus</u>	P°	P	P	P	P°	P°	P°	P	P°	P°
<u>Penicillium spp.</u>	P°	P	P	P	P°	P°	P°	P	P°	P°
<u>Cladosporium eladosporioides</u>	P	P	P	P	P	P	P	P	P	P
<u>Trichoderma viride</u>	P	P	P	P	P	P	P	P	P	P
<u>Fusarium aqueductum</u>	P	P	P	P	P	P	P	P	P	P
<u>Mucor alternans</u>	P	P	P	P	P	P	P	P	P	P
<u>Alternaria tenuis ss. lat.</u>	P	P	P	P	P	P	P	P	P	P
<u>Aspergillus niger</u>	P	P	P	P	P	P	P	P	P	P
<u>Candida parapsilosis</u>	P	P	P	P	P	P	P	P	P	P
<u>Aspergillus ochraceus</u>	P	P	P	P	P	P	P	P	P	P
<u>Geotrichum candidum</u>	P	P	P	P	P	P	P	P	P	P
II										
<u>Candida krusei</u>	P	P	P	P	P	P	P	P	P	P
<u>Verticillium lateritium</u>	P	P	P	P	P	P	P	P	P	P
<u>Candida spp.</u>	P°	P°	P°	P°	P°	P°	P°	P	P°	P°
<u>Hansenula californica</u>	P	P	P	P	P	P	P	P	P	P
<u>Kloeckera apiculata</u>	P	P	P	P	P	P	P	P	P	P
<u>Achlya spp.</u>	P	P	P	P	P	P	P	P	P	P
<u>Dematiaceae spp.</u>	P°	P°	P°	P°	P°	P°	P°	P	P°	P°
<u>Rhizopus spp.</u>	P°	P°	P°	P°	P°	P°	P°	P	P°	P°
<u>Aphanomyces sp.</u>	P°	P°	P°	P°	P°	P°	P°	P	P°	P°

TABLE 17b (Continued)

Habitat types		A	O	B	V	S	AP	RB	PT	U	U
Constancy	Sample sites	3	10	2	12	5	3	3	3	4	1
Class	Number of samples	3	10	3	19	10	6	3	4	8	1
	<u>Aspergillus flavus</u>	P		P	P	P	P				
	<u>Penicillium lilacinum</u>			P	P	P	P				
	<u>Rhodotorula texensis</u>	P									
	<u>Aureobasidium pullulans</u>		P	P	P	P	P			P	P
	<u>Rhodotorula mucilaginosa</u>		P	P	P	P	P	P	P		
	<u>Gliocladium spp.</u>		P ^o	P ^o	P ^o	P				P	
	<u>Fusarium oxysporum</u>		P	P	P	P					
III	<u>Rhodotorula glutinis</u>	P	P		P						
	<u>Phoma spp.</u>	P ^o			P	P ^o	P ^o	P ^o	P	P ^o	
	<u>Cryptococcus laurentii</u>	P			P	P	P	P			
	<u>Gliosporium sp.</u>	P ^o			P	P ^o	P ^o				
	<u>Stemphylium sp.</u>	P ^o			P	P ^o	P ^o			P	
	<u>Mucor hiemalis</u> sect.	P			P	P		P			
	<u>Paeecilomyces varioti</u>	P			P	P					
	<u>Penicillium funiculosus</u>	P			P	P					
	<u>Candida tropicalis</u>			P	P	P					
	<u>Trichosporon cutaneum</u>	P			P	P			P	P	
	<u>Aspergillus spp.</u>	P			P	P ^o					
	<u>Torulopsis fasmata</u>				P	P					
	<u>Cephalosporium spp.</u>	P			P	P ^o	P ^o	P ^o	P	P ^o	
	<u>Epicoecum nigrum</u>			P	P	P					
IV	<u>Torulopsis glabrata</u>	P	P				P				
	<u>Curvularia lunata</u>	P			P		P			P	
	<u>Rhizopus arrhizus</u>	P			P		P				
	<u>Rhodotorula spp.</u>	P			P		P				
	<u>Aspergillus flavipes</u>	P			P		P				
	<u>Aspergillus tamaris</u>	P			P		P				
	<u>Candida intermedia</u>		P		P						

TABLE 17b (Continued)

Habitat types	A	O	B	V	S	AP	EB	PT	U	S
Constancy	3	10	2	12	5	3	3	3	4	1
Class	3	10	3	19	10	6	3	4	8	1
<u>Trichosporon sp.</u>	P*			P*						
<u>Cryptococcus diffluens</u>			P	P	P	P	P	P		
<u>Fusarium spp.</u>			P	P	P					
<u>Penicillium velutinum</u>			P	P	P					
<u>Stachybotrys atra</u>			P	P	P					
<u>Rhizolpidium sp.</u>			X	X	X					
<u>Alloscyces sp.</u>			X	X	X					
<u>Fusarium solani</u>			P	P	P					
<u>Myrothecium torridum</u>			P	P						
<u>Memnoniella echinata</u>	P									
<u>Verticillium sp.</u>					P					
<u>Absidia spinosa v. biappendiculata</u>			P				P			
<u>Candida utilis</u>				P	P					
<u>Cryptococcus albidus</u>	X			P	P					
<u>Candida pulcherrima</u>	X			P	P					
<u>Torulopsis inoospicua</u>	X									
<u>Paeclomyces sp.</u>										
<u>Saccharomyces cerevisiae</u>		P								
<u>Torulopsis holmii</u>		P								
<u>Trichosporon maritimum</u>		P								
<u>Conidiobolus villosus</u>		P								
<u>Penicillium variabile</u>		P								
<u>Torulopsis candida</u>			P							
<u>Torulopsis stellata</u>			P							
<u>Absidia ramosa</u>			P							
<u>Rhizopus stolonifer</u>			P							
<u>Candida pelliculosa</u>			P							
<u>Candida pseudotropicalis</u>				P						
<u>Candida ?solani</u>				P						

TABLE 17b (Continued)

Habitat types	A	O	B	V	S	AP	FB	PT	U	S
Constancy	3	10	2	12	5	3	3	3	4	1
Class	3	10	3	19	10	6	3	4	8	1
<u>Candida ?zeylanoides</u>				P						
<u>Pichia membranefaciens</u>				X						
<u>Torulopsis aeria</u>				X						
<u>Saprolegnia sp.</u>				X						
<u>Actinomyces elegans</u>				X						
<u>Syncephalastrum racemosum</u>				P						
<u>Aspergillus terreus</u>				P						
<u>Aspergillus terreus v. floccosus</u>				P						
<u>Aspergillus versicolor</u>				P						
<u>Coniothyrium sp.</u>				P						
<u>Fusarium culmorum</u>				P						
<u>Fusarium moniliforme v. subglutinans</u>				P						
<u>Aspergillus wentii</u>				P						
<u>Fusarium acuminatum</u>				P						
VI										
<u>Cryptococcus luteolus</u>						P				
<u>Fusarium avenaceum</u>						P				
<u>Mucor petropoli-tanus sect.</u>						X				
<u>?Ascomycete</u>							X			
<u>Rhodotorula graminis</u>								P		
<u>Rhodotorula pennaeus</u>								P		
<u>Aspergillus sydowii</u>								P		
<u>Cladosporium herbarum</u>								P		
<u>Candida guilliermondii</u>										
<u>Rhizopus chinensis group</u>										
<u>Aspergillus nidulans</u>										
<u>Chaetomium sp.</u>										
<u>Scoptulariopsis sp.</u>										

Symbols: P--lymphophile

X--Lysazene

*no history of pollution in habit

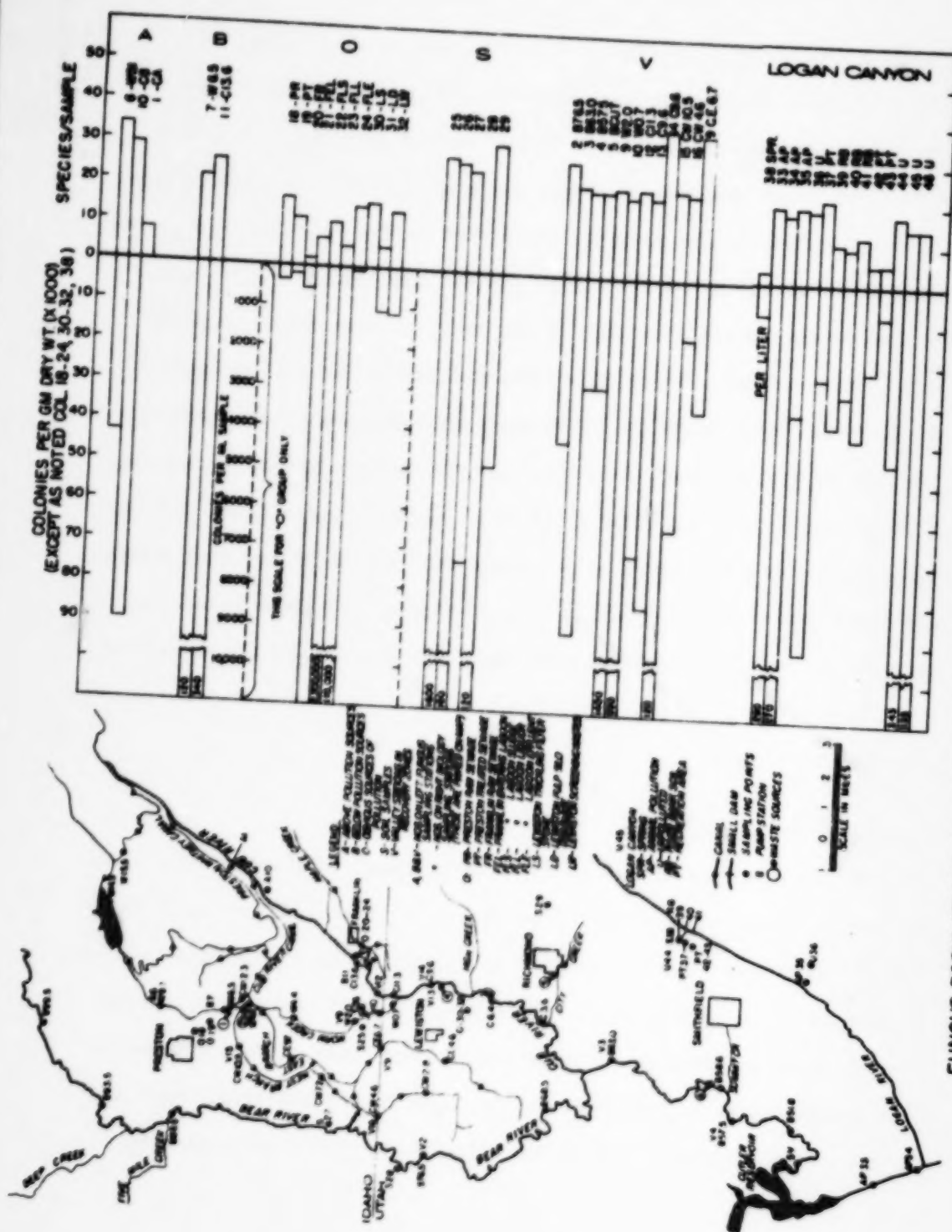
Table 18. Numbers Of Fungal Colonies Recovered From Habitat
Types By Plate Count Methods
(Autumn Survey)

Code Habitat type	Number of Samples	Total species	Estimated average number of colonies per volume of sample
Cache Valley			
A Above sources of pollution	3	44	38,000/g
O Obvious sources of pollution	10	44	270,000/ml
B Below sources of pollution	2	35	220,000/g
V Valley streams after absorbing pollution	12	77	209,000/g
S Soils (mostly agricultural, irrigated or not, sometimes with polluted water)	5	50	402,000/g
Logan Valley			
AP Animal pollution (pasture or grazing)	3	37	200,000/g
RB River bank (no domestic livestock)	3	19	34,000/g
PT Path and toilet area (dry)	3	23	18,000/g
S Spring water (Rick's Spring)	1	3	8 2/L
U Unpolluted (apparently) by man or livestock	4	32	250,000/g

A map of the Cache Valley and the Logan River is given in Figure 4, with sampling points indicated. Figure 4 includes 6 sets of bar graphs showing the number of fungus species from all media, and colonies of fungi observed on plates for each sample location by habitat types. Each bar in the graph is identified by the code number used for the biological survey of the stream and the code number for the mycological study. Where sampling did not overlap, only the mycological code number is used. These code numbers refer to the station numbers on the map. Three techniques were used for reporting colony numbers. In the case of spring water, numbers of species and colonies are given per liter of sample although only about 60 ml of water was sampled. In the case of sewages and sludges (S), numbers of colonies of isolated fungi are indicated according to milliliter of sample. If these values had been converted to colonies per gram dry weight, they could have reached the billion level. In all other cases, samples were corrected to colonies per gram dry weight.

Constancy

In Table 17b are listed the species of fungi obtained in this study. The six constancy classes listed are based on species occurring in Cache Valley. Constancy Class I includes 15 species or species groups that are present in all 5 habitat types. Constancy Class II includes 16 species present in only 4 of these habitat types. For Class I species there is also a high degree of constancy in the Logan Canyon habitats. Most of the species in Classes I and II are ubiquitous. Those not appearing in



FUNGUS POPULATIONS AT SELECTED SAMPLING SITES BEAR RIVER AND TRIBUTARIES
FIGURE 4

Logan Canyon may be present, but were missed in selection of sampling sites, or through some other factor such as insufficient number of samples. Many of the 14 species in Class III are usually ubiquitous, but habitat requirements may be such that sampling techniques used were inadequate for their complete representation. Constancy Class IV represents those 16 species found in only two of the habitat types sampled in the Cache Valley. Some of them were also found in Logan Canyon. Class V represents 35 species isolated from only one habitat type, more species in this group being found in the lower valley stations than in other locations. Only 5 of these were also found in Logan Canyon samples. Class VI includes those 13 species isolated only from Logan Canyon samples.

Fidelity

As used here, fidelity is defined as the percentage of species in one habitat occurring in adjacent habitats. To compare the populations of organisms found in contiguous associations, Cooke (1955) used a fidelity chart based on the percentage of organisms found in each combination of associations in a study region. In applying this technique to the data obtained here, Figures 5 and 6 were developed. Each of the habitat types used in these charts is the same as those used in Table 17.

Total Population

Many different criteria are available for determining the total number of organisms present in a sample or a habitat. The most commonly used is the direct observation of the population. For different segments of a population, different numbers of observation times are required.

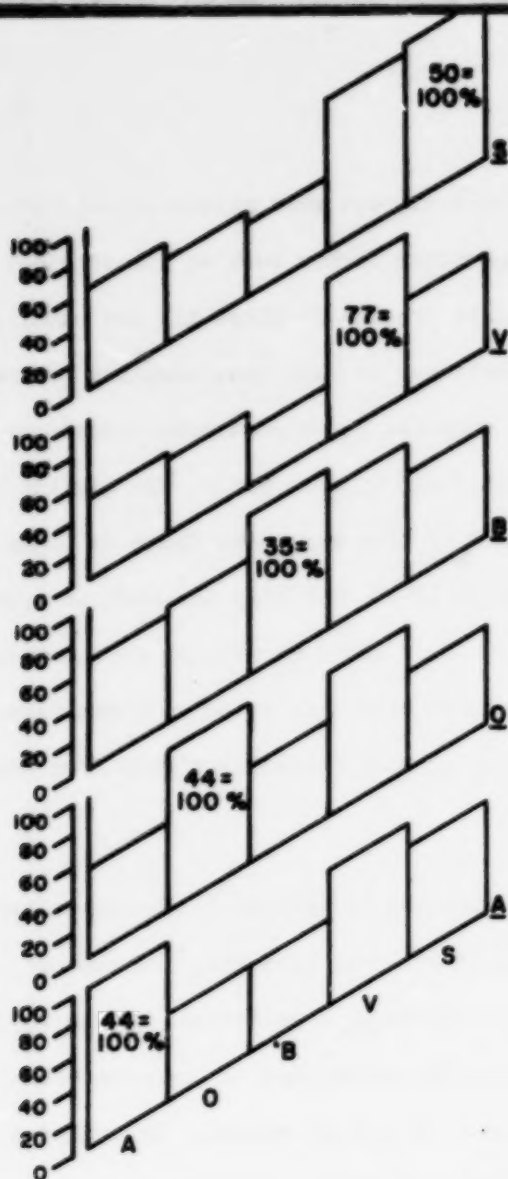


FIGURE 5

Fidelity Chart. Cache Valley Stations. Each group of stations in five basic habitat types is compared with the other on the basis of the percentage of species in one habitat type occurring in adjacent habitat types.

Letter code: A - above pollution sources; O - obvious pollution sources; B - below pollution sources; V - valley stations; S - soils.

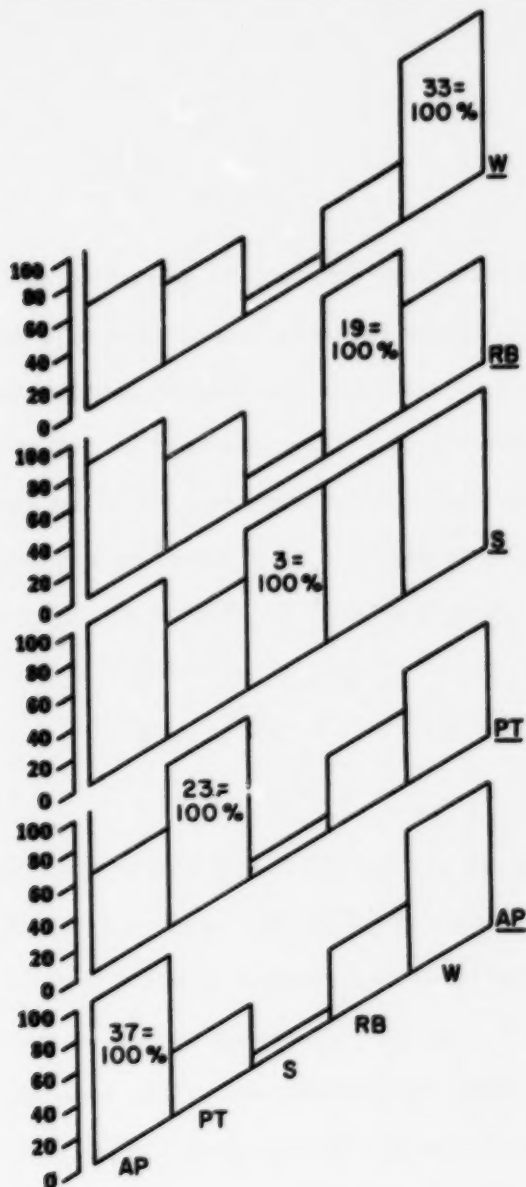


FIGURE 6

Fidelity Chart. Logan River Samples. Each group of samples in five basic habitat types is compared with the other on the basis of the percentage of species in one habitat occurring in adjacent habitat types. Letter code: AP - animal pollution; PT - soils in pathway and around toilet doors; S - spring water; RB - river bank soil; U - apparently unpolluted areas.

Fifteen to 20 quadrants or plots may be needed to define a vascular plant association, or the bacterial population of a highly polluted stream may require no less than 200 sets of samples to satisfy questions about the total population. If we assume that 50% of a total population is recorded in the first observation of a sampling area (M. Lange, 1948), 80% in four, and 85 to 97% in 6 to 8 observations, and if these percentages are transferred to soil or similar samples, we arrive at an estimated minimum number of species available for study in the Cache and Logan Valleys (Table 9).

Problems in Quantification

In Table 9 are listed the five habitat types into which samples from each of the two portions of the Cache Valley study were divided. These habitat types are briefly characterized, and the number of samples obtained from each is noted. It should be mentioned that only one sample was obtained from each of the habitats within each habitat type. The results of colony isolation are averaged, and the number of species listed is cumulative for each habitat type. The results of colony counts apply to only those colonies and species obtained on neopeptone - dextrose - rose bengal agar. The number of species is that obtained from all media and isolation techniques used. Since some species present may have had nutrient requirements not met by the few media used, the number of species listed may be short of the possible total. The species represented on the agar plates from which colony counts were obtained represent only between one-half and two-thirds of the total list of species. It is not

Table 19. Minimal Populations Of Soil Fungi In Cache Valley

<u>Number of observations</u>	<u>Theoretical % of Total</u>	<u>Cumulative Total</u>
1-3	less than 50	109 actually observed
4	80	174
6	90	196
8	95	207
	100	218

certain that colonies that were counted were produced by mycelial fragments embedded in or adherent to fragments of soil or organic matter, or whether they were produced only by single spores or by a combination of both types of disseminules. Thus, because more species were present, and some disseminules of those that were present may have been inhibited by competition, crowding, antibiosis, toxicity of rose bengal, or some other factor, the colony counts obtained are probably low and indicate that a larger number of spores or viable fungus cells were present in a sample. In addition, a more accurate idea of the numbers of viable fungus cells in a given habitat will be obtained by repeated sampling over a limited period of time within a season or at intervals throughout the year.

Discussion

Through the use of three techniques and five media, 109 species or species groups of fungi were recovered from samples of water, soil, sewage, and related materials obtained during a survey of the pollution of the Bear River in Idaho and Utah. These records included a number of species of yeasts.

Of these species, 15 were present in all five major habitat types in both the polluted Cache Valley samples and the relatively clean Logan River samples. Relatively few species were restricted to any one of these habitat types, and 41 species were found only once in any habitat type within either of the two river systems. Six classes of species were recognized on the basis of occurrence in decreasing numbers of habitat types in the Cache Valley, and the sixth included a group of species found

only in the Logan River Valley; of these, two were found twice, the remainder only once.

Attempts were made, on the basis of the numbers of species of fungi of all kinds isolated from each habitat type, to correlate the occurrence of fungal species with the different types of habitat in which they occurred. When the coefficient of similarity was applied to the set of data obtained, and a two-dimensional ordination was drawn, the results indicated that insufficient data were available for an analysis of the information. Additional samplings throughout a longer period of time appeared to be required. The fidelity chart appears to be a promising technique for demonstrating differences between habitats in a study of this type. However, in this case, too, it seems possible that too few samples were processed to produce results on which too much reliance can be placed. If sufficient samples had been processed to yield more than a potential 50% of the population (4 sets from as many seasons), or better, at least 75% of a population at each sampling site (6 or 8 sets), more reliance could be placed on the data that were developed.

The apparent lack of clear-cut distinctions between the five habitat types considered here may be related to certain principles summarized by Gleason (1939). In his individualistic concept of the plant association, Gleason considers that each community of organisms is composed of a number of individual species, each having reproductive powers beyond their needs, each having special methods of migration, and each responsive simultaneously to regular and predictable variations in environmental influences, irregular and unpredictable influences in

environmental influences, and cumulative environmental changes. The development of a biotic unit depends either on the appearance of new ground, or on the disappearance of an existing association. In this process of development, each organism seizes and uses the particular time-period during which the environment is in a condition suitable to it. At the same time, the continuation at any point of a biotic association results from interaction of available population and environmental selection. As a result of this environmental selection, the biota varies constantly in time and continuously in space.

The five habitat types to which the Cache Valley stations have been assigned are: A - above the obvious sources of pollution; O - the obvious sources of pollution; B - below the obvious sources of pollution; V - Cache Valley stations in the several affected streams in areas which might be referred to as "zones of recovery"; and S - various stations from which soil samples were obtained in dry-land farming areas, grazing areas, and areas subject to irrigation where irrigation water was taken from either polluted or unpolluted reaches of the studied streams. From the list of species in Table 1 it is obvious that a number of fungal species occurred in the clean water upstream from the sources of pollution, other species are definitely associated with the polluting substances which entered the streams at the obvious sources of pollution. Both types of fungi continued as members of the population as the polluting substances were diluted out below the obvious sources of pollution, and as further dilution took place in the Valley stations additional species of fungi were able to become members of the population of the changed environments.

Fungi are organisms which reproduce by the production of excessively large numbers of spores, given the proper conditions, or by the production of large numbers of budding cells, or of large masses of cells joined together in, or members of, long filaments capable of being fragmented by various natural agents such as turbulent stream conditions, natural fragmentation, partial eating by members of the animal population, and other factors. Spores, cells, and mycelial fragments may be carried by air or water from the place of production to a suitable location for growth as determined by environmental factors. Most important of these environmental factors are sources of nutrients and the presence of organic pollutants in stream water, and in sludges resulting from excess pollutants in the water, form an ideal source of nutrients for these fungi. From constancy classes V and VI in Table 17b it is apparent that not all fungi in any given location are capable of adapting themselves to an aquatic habitat such as those represented by sampling sites grouped together in habitats A, B, O, and V. However, those which are capable of becoming adapted to such environments develop communities which can be studied fairly readily. These fungi are probably capable of surviving regular and predictable, as well as irregular and unpredictable, changes in the environment such as extremes of heat and cold, drought and excessive precipitation. There is probably little problem to the organism in the latter circumstances because the levels of the water in the streams studied are rigidly controlled by the irrigation system in use in the Valley. However, the continual release of organic pollutants from sugar beet processing plants, canneries, and dairies, as

well as from community sewage systems, provides a series of cumulative environmental changes through which these fungi have had to live for a number of years. These changes, at the times when they became effective, provided the time-period during which the environment became sufficiently altered to permit the colonization of new additions to the population and their development. The new additions to the population had to contend with an already present community of fungi at any given point. This is evident from the fact that communities are established at all the clean water stations above the sources of pollution. The results of the interaction between populations already present and additions to populations brought with the polluting substances or responsive to these substances, are noted in constancy classes II, III, and IV in Table 17b. Because of the few samples studied in this series, little can be said about the effect of environmental selection in terms of constant variation in time, although I have made certain inferences concerning this above. However, columns A, O, B, and V in Table 17b. indicate that the fungus biota varies continuously in space from each other. In addition, where soils are irrigated with organically enriched water they tend to approach the Valley stations in similarity of population, forming a somewhat bifurcate type of community continuity.

It is interesting to note that as streams recovered from the shock of the sudden increase in organic pollution, fungus populations increased. Based on total numbers of species listed in Table 17b, in the streams above the sources of pollution a total of 44 species was recovered. At the obvious sources of pollution this number dropped to 40, but increased

to 44 after initial mixing with stream water below the obvious sources of pollution. Further downstream, at the several Valley stations, the total number of fungal species recovered increased to 77. The results of the autumn survey of these areas are reported in Table 2. Here the total number of species is the same for the upper two sets of stations, but the number of species for the stations below the obvious sources of pollution decreased to 35 possibly indicating a certain amount of toxicity resulting from the sugar beet campaign and its increased pollution load. Further down the stream, the Valley stations again show a total of 77 species or species groups of fungi recovered. A somewhat different pattern is shown by the estimated average number of colonies per volume of sample used in plating procedures. This indicates that while the Valley samples have a broader species spectrum, increased competition for less and less food materials results in a smaller total population.

It appears that in the Cache Valley, of the larger number of fungi found in the soil, on creek banks, in bottom sediments in streams, and in similar habitats, the principal species found are lymaphiles or lymaxenes (see Chapter XI, B. for definitions). Here the larger portion of pollution is seasonal as a result of man's activity in food processing industries. Either lymabionts have not yet become established, the conditions for establishment do not occur, or the sampling techniques used have not yet picked them up. Lymaphobes may be represented by a few species that occur in the Logan Canyon Sampling sites, but most of the species represented in only one habitat-type have been found elsewhere in more heavily polluted sites.

D. The Cache la Poudre River*

Geography

The Cache la Poudre River originates in Poudre Lake (elevation 10,752 ft.) in the Rocky Mountains of Colorado on the east side of the Continental Divide near Milner Pass, Rocky Mountain National Park. The lake has considerable recreational value because it is well stocked with fish and is followed for its length by Trail Ridge Road (U. S. Highway 34), the only trans-mountain highway across the Park. From Poudre Lake, the Cache la Poudre River flows in a generally northern direction, draining a long valley that is relatively inaccessible. A tributary brings water from Barnes Meadow Reservoir from which water has been brought by a small canal from Chambers Lake at or near the head of the Laramie River. Further downstream a tunnel brings additional water to the Cache la Poudre River directly from the Laramie River. Camp grounds and occasional summer homes attest to man's use of these watersheds. At about a point called Spencer Heights (marked by a small grocery and supply shop) along Colorado State Route 14, the river turns northeastward and later almost due eastward through a canyon in which are found, as the topography permits, summer homes, camp grounds, group camps, and other recreational areas, as well as weak attempts at mountain farming; these increase as the river descends the canyon. The broad flood plain of the river supports increased farming as the river leaves the canyon and the mountains and starts its journey across the Great Plains of Colorado. Bellvue and Laporte are farm communities upstream from Fort Collins through which the Cache la Poudre River flows. Just below Bellvue the river receives water from Horsetooth Reservoir, a large lake to which

* Reproduced with permission from: Mycopathologia et Mycologia Applicata 35: 361-372, 1968.

water from the Big Thompson Project has been brought from the Colorado River Basin through a series of storage dams and pumping stations and stored for irrigation usage. The lakes and streams of this system also are used, sometimes heavily, for all types of recreation.

Downstream from Bellvue, the Cache la Poudre River receives a variety of effluents. Above Fort Collins these are relatively light since the communities are small, served by septic tanks or privies, and there is little industry except agriculture. At and below Fort Collins the stream receives the effluents of various industries, such as aggregate production (sand and gravel washings), meat processing plants, and a municipal treatment plant of low quality. Further downstream, in addition to runoff from fields and feed lots, the stream receives the effluents from a sugar beet processing plant. The Cache la Poudre River joins the South Platte River just downstream from Greeley, Colorado.

Technique

In a survey of the biota of the Cache la Poudre River to determine the effects of polluting substances on the biota and the effects of the biota on the polluting substances, Lowell Keup, on October 25, 1964, took a set of samples at five stations between Bellvue and Greeley and returned them to Cincinnati for processing in the Fungus Studies Laboratory. On July 22 and 23, 1965, Dr. L. W. Durrell and the writer obtained a second set of samples at these five stations, and took a first set from five stations upstream from Bellvue. Two of these sets were from the headwaters of the Cache la Poudre River, the others from Spencer Heights to below the Fort Collins water filtration intake.

Five types of material were sampled. In the first set of samples, taken by Keup when the water was at about its lowest autumn flow, sampling at each station included water in the flowing stream, sediment in the stream bottom, bank soil at the interface of the water and the soil, floodplain soil subject to occasional overflow, and upland soil subject to overflow of the stream only at deepest flood stages. When the second set of samples was obtained, the river was swollen with summer rainwater at most points and only four types of samples were collected: water, bottom sediment, stream-bank interface, and upland soil. Because of the higher water, the bottom sediment of the second sampling possibly corresponded with the stream-soil interface samples of the first, and the stream-soil interface samples of the second period may have corresponded with the flood-plain samples of the first.

Using techniques outlined above, from each type of sample pour plates were prepared on which colonies were counted and from which colonies of unrecognized species of fungi were picked, hemp seed bait plates were prepared, and yeasts were developed in the shaken flask technique.

Results

Colony counts on poured plates of samples collected in both autumn (October) and summer (July) are given in Table 20. In general, in the five downstream locations, larger numbers of colonies appeared in the October than in the July samples. Two factors may account for this apparent discrepancy. First, July samples were obtained when the river was carrying heavy runoff from upstream. This serves as a scouring action

Table 20. Fungus Colonies in Cache la Poudre River Samples

Station	Season	Location	Water	Sediment	Habitat Bank Soil	Upland Soil
1	F S	Poudre Lake	52,000	5,200	4,800	16,400
2	F S	Cache la Poudre River at mouth of Poudre Lake	15,000	2,600	4,300	89,000
3	F S	Spencer Heights	17,000	2,800	1,500	544,000
5	F S	Below Fort Collins water filtration plant	13,000	7,600	1,600	19,000
6	F S	Bellvue	83,000 16,000	205,000 123,000	88,000 2,900	92,000 9,400
7	F S	Below Fort Collins Sewage Treatment Plant	7,800 13,000	280,000 4,000	4,600 1,100	20,000 75,000
8	F S	Near Windsor, above sugar plant	1,200 8,400	2,500 1,900	45,000 6,600	25,000 23,000
9	F S	Near Bracewell, below sugar plant	22,000 9,600	180,000 3,700	44,000 2,600	34,000 22,000
10	F S	Spanish Colony	15,000 6,800	180,000 4,500	11,000 2,000	34,000 17,000

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which removes sediments and sludges from the stream bottom and those portions of the inundated floodplain that were sampled. Second, in the lower parts of the stream, the pollutants resulting from seasonal processing of such plant products as beet roots had not yet started in July, but was in full progress in late October.

The kinds of fungi isolated by the several techniques used are listed in Table 21. This includes 128 species of filamentous soil molds, water molds, and yeasts. The numbers of species isolated from each habitat in each location, are summarized in Table 22. The occurrence of yeast species is summarized in Table 23. Since some isolates picked from plates were not completely identified to species, partly because of lack of sporulation, loss of the culture before completion of the analysis, or insufficient growth details for diagnosis, a number of categories appear in Table 21 as "sp." or as "unidentified molds", etc. Including these incompletely identified or unidentified groups, eight species or species groups were present at all 9 stations. While some species are common to each of the five habitats sampled at each station, many species are restricted in our present experience to one, two, or three of these habitats. Possibly with more frequent and thorough sampling some of the gaps may be filled. The species list is summarized in Table 24 where the total number of times the species was observed in all stations and habitats from which it was isolated are given.

Note that in the four stations at higher elevations, fewer species were recovered. This may have resulted from the fact that only one set of samples from these stations was processed, or that the apparent pollution levels were lower at each station except at station 1. Where more samples were studied, larger numbers of species were recovered; this may

Table 21. Distribution of fungal species in Cache la Poudre River Basin

Species	1	2	3	5	6	7	8	9	10
<i>Penicillium lilacinum</i>	WSB U	WSB U	WSB U	WSB U	WSB*U	WSB	WSB U	WSB U	W B U
Unidentified molds	WSB U	WSB U	SB U	WSB U	WSB*U	WSB*U	WSB*U	WSB*U	WSB U
<i>Phialophora jeanselmei</i>	WSB U	WSB U	S	S	WSB	WSB*U	WSB*U	WSB*U	WSB U
<i>Cephalosporium</i> spp.	WSB	W B U	W	B	WSB	SB*U	WSB*U	WSB U	WSB U
<i>Fusarium oxysporum</i>	W	B U	B U	B U	SB*U	S U	B U	WSB*U	W U
<i>Trichoderma viride</i>	W B	U	U	WSB U	WSB*U	WSB*	SB*U	SB*U	WSB U
<i>Penicillium</i> spp. indet.	SB U	SB U	B U	WSB U	WSB*U	WSB*U	WSB U	WSB U	WSB U
Unidentified yeasts	S	S U	W B U	SB	WSB*U	WSB U	WSB U	WSB*U	WS U
<i>Phoma</i> spp.	B	-	W B U	W B U	S U	W *	S	SB U	WS*U
<i>Gliocladium roseum</i>	W	-	B	U	B*U	*U	WSB*U	B*U	SB*U
<i>Rhodotorula</i> sp. indet.	U	B U	-	B	S	U	W *U	WS U	S
<i>Mucor hiemalis</i>	B U	B U	-	WSB U	SB	B	S U	B U	B U
<i>Mucor</i> spp. indet.	SB U	B	-	WS U	SB*U	SB*U	SB*U	SB*U	WSB*U
<i>Penicillium funiculosum</i>	W	W	-	B	WSB U	B	B	SB	S
<i>Aspergillus niger</i>	SB U	S	-	S	S U	*	U	-	B U
<i>Penicillium corymbiferum</i>	S	S	-	S	B	WSB U	S	-	WS U
<i>Mucor alternans</i>	U	B U	-	-	W *	W	W B*U	-	-
<i>Fusarium roseum</i>	WS	W	WS	W	B*	-	-	-	S U
<i>Epicoecum purpurascens</i>	B	-	U	W	B	-	-	-	W B*
<i>Pestalotia heteroxcornis</i>	B	-	W	W	B	-	-	-	-
<i>Penicillium expansum</i>	B	-	-	SB U	-	B	-	S	-
<i>Penicillium rubrum</i>	S	-	-	W	-	B *	-	S	S
<i>Trichoderma alba</i>	B	-	-	S	B U	-	-	W U	-
<i>Penicillium purpurosogenum</i>	B U	-	-	-	-	-	-	-	-
<i>Aspergillus utus</i>	WS	-	-	-	S *U	-	B	W B U	*
<i>Alternaria tenuis</i>	W	-	-	-	*U	-	U	S	*
<i>Penicillium nigrificans</i>	U	-	-	-	B	-	-	-	-
<i>Moniliella echinata</i>	B	-	-	-	-	S	-	-	-
<i>Cryptococcus luteolus</i>	S	-	-	-	-	-	B	-	-
<i>Actinomyces elegans</i>	S U	-	-	-	-	-	-	-	-
<i>Aphanomyces</i> sp.	-	-	-	-	-	-	-	S	B*
<i>Cladosporium cladosporioides</i>	-	W U	W	W U	W	W	WS U	B U	-
<i>Aspergillus fumigatus</i>	-	W	-	S	S *	WS *U	WSB*	WSB	W B*U
<i>Trichosporon</i> spp. indet.	-	B	-	W	S	S	W	-	W
<i>Aspergillus flavus</i>	-	B	B	-	-	S	W *	WS	W
<i>Oidiodendron</i> sp.	-	W	W	-	-	W	-	W B	W
<i>Torulopsis farnata</i>	-	B	WSB	U	-	-	-	-	-
<i>Penicillium implicatum</i>	-	B	-	B	-	-	-	-	-
<i>Beauveria bassiana</i>	-	B U	-	-	-	W	-	-	-
<i>Sepedomium</i> sp.	-	B	-	-	-	-	-	-	-
<i>Verticillium</i> sp.	-	B	-	-	-	-	-	-	-

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<i>Aspergillus versicolor</i>	-	-	W	U	U	B	W *	B	W B	WS *
<i>Rhodotorula mucilaginosa</i>	-	-	W	U	BU	*U	WB	B	-	SB*U
<i>Aspergillus ochraceus</i>	-	-	W	B	-	S U	W	S	SB U	SB
<i>Geotrichum candidum</i>	-	-	W	-	-	B	WS*	B*U	WS	SB
<i>Aureobasidium pullulans</i>	-	-	W	-	-	W	U	U	SB	-
<i>Verticillium lateritium</i>	-	-	W	-	-	-	S *	*	WSB*	W B*
<i>Mucor plumbeus</i>	-	-	U	U	WS	S	-	-	-	-
<i>Achlya klebsiana</i>	-	-	U	U	-	-	-	-	SB	-
<i>Cryptococcus diffluens</i>	-	-	B	U	-	-	-	-	-	-
<i>Penicillium aurantiacum</i>	-	-	U	-	-	-	-	-	-	-
<i>Penicillium puberulum</i>	-	-	SB	-	-	-	-	-	-	-
<i>Rhizopus</i> spp.	-	-	-	S U	-	BU	B	SB	*U	*U
<i>Candida krusei</i>	-	-	-	B	-	-	WB	SB	WS	SB
<i>Rhizopus arrhizus</i>	-	-	-	S	-	-	-	U	SB U	SB U
<i>Penicillium variabile</i>	-	-	-	B	-	-	W	-	BU	S U
<i>Rhizopus chinensis</i> sect.	-	-	-	S	-	-	U	-	-	S
<i>Fusarium aqueductum</i>	-	-	-	W	-	W	-	-	B	W
<i>Penicillium simplicissimum</i>	-	-	-	W	-	B	-	-	U	-
<i>Stachybotrys atra</i>	-	-	-	U	U	U	-	-	W B U	SB*
<i>Paecilomyces marquandii</i>	-	-	-	U	-	*	B	-	-	W U
<i>Cunninghamella elegans</i>	-	-	-	-	-	S U	W	B	B	-
<i>Rhodotorula glutinis</i>	-	-	-	-	-	S	U	S	WS	U
<i>Stemphylium consortiale</i>	-	-	-	-	-	*	U	U	B	WS *
<i>Fusarium</i> spp. indet.	-	-	-	-	-	SB U	W	S*U	U	W B *
<i>Aspergillus ventii</i>	-	-	-	-	-	*	-	B	S U	B
<i>Paecilomyces varioti</i>	-	-	-	-	-	BU	WSB	-	-	S
<i>Rhodotorula texensis</i>	-	-	-	-	-	SB	S	-	SB	-
<i>Penicillium oxalicum</i>	-	-	-	-	-	S	WS	-	-	S *
<i>Cryptococcus laurentii</i>	-	-	-	-	-	*	-	B	-	WS U
<i>Penicillium janthinellum</i>	-	-	-	-	-	S	-	W B	W	-
<i>Penicillium martensii</i>	-	-	-	-	-	U	-	-	S	-
<i>Candida</i> spp. indet.	-	-	-	-	-	U	-	-	-	-
<i>Gliomastix convoluta</i>	-	-	-	-	-	S	B	-	S	-
<i>Myrothecium verrucaria</i>	-	-	-	-	-	*	*U	-	-	-
<i>Candida utilis</i>	-	-	-	-	-	-	-	-	-	-
<i>Penicillium javanicum</i>	-	-	-	-	-	B	-	-	-	-
<i>Penicillium sclerotiorum</i>	-	-	-	-	-	B	-	-	-	-
<i>Torula</i> sp. indet.	-	-	-	-	-	S	-	-	-	-
<i>Torulopsis aeria</i>	-	-	-	-	-	*	-	-	-	-
<i>Torulopsis inconspicua</i>	-	-	-	-	-	B	-	-	-	-

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<u>Ascomycete</u>	-	-	-	-	-	WS
<u>Aspergillus nidulans</u>	-	-	-	-	-	*
<u>Aspergillus sydovii</u>	-	-	-	-	-	W
<u>Candida intermedia</u>	-	-	-	-	-	S
<u>Curvularia lunata</u>	-	-	-	-	-	*
<u>Helminthosporium sp.</u>	-	-	-	-	-	*
<u>Phialocephala heterospora</u>	-	-	-	-	-	S
<u>Stilbella bulbicola</u>	-	-	-	-	-	W
<u>Xylaria sp.</u>	-	-	-	-	-	W
<u>Allomyces arbusculus</u>	-	-	-	-	-	U

FOOT NOTE:

W - Water
 S - Bottom sediment
 B - Bank soil
 * - Floodplain Soil
 U - Upland soil

For habitat location data, see Table 1.

Table 22.

Number of species at each sampling station by habitat

Habitat	1 Head of Poudre Lake	2 Poudre River below Lake Outlet	3 Spencer Height	5 Below Water Filtration Plant Poudre Canyon	Station 6 Bellvue	7 Fort Collins below sewage treatment plant	8 Windsor	9 Bracewell	10 Spanish Colony
Running Water in River	11	9	13	15	12	28	14	25	32
Sediment in bottom of stream	14	7	6	18	27	24	22	36	33
Soil from bank at interface of water	14	18	12	16	29	23	31	36	27
Upland Soil, from above usual high water level (Flood Plain Soil)	13	13	10	18	24	23	27	35	30
Total species at each Station	32	37	29	37	21	20	15	12	20
					37	62	55	68	64

Table 23. Occurrence of Yeasts in Cache la Poudre River

Species	October	July	Total Number of Stations
<u>Candida curvata</u>		*	1
<u>intermedia</u>	*		1
<u>krusei</u>	*	*	4
<u>lambica</u>	*	*	7
<u>parapsilosis</u>		*	2
<u>tropicalis</u>	*		1
<u>utilis</u>	*		1
spp.		*	2
<u>Cryptococcus diffluens</u>		*	1
<u>laurentii</u>	*		3
<u>luteolus</u>	*	*	2
<u>Prototheca</u> sp.	*		1
<u>Rhodotorula glutinis</u>	*		5
<u>mucilaginoso</u>	*	*	6
<u>pennaeus</u>	*		1
<u>texensis</u>	*		3
spp.	*	*	7
<u>Torulopsis aerea</u>		*	1
<u>famata</u>		*	3
<u>inconspicua</u>	*		1
<u>versatilis</u>	*		1
spp.	*	*	2
<u>Trichosporon cutaneum</u>	*	*	2
<u>pullulans</u>	*		1
spp.	*	*	4
Unidentified	*	*	9

Table 24. Fungi observed in isolations from the Cache la Poudre River, Colorado

Species	Observation number	Number of Habitats (of 42)	Number of Stations (of 9)
Unidentified filamentous fungi	275	40	9
<u>Penicillium</u> spp.	132	35	9
<u>Trichoderma viride</u>	98	29	9
<u>Mucor</u> spp.	72	28	8
Unidentified white yeasts	65	29	9
<u>Penicillium lilacinum</u>	49	36	9
<u>Fusarium oxysporum</u>	38	19	9
<u>Cephalosporium</u> spp.	36	26	9
<u>Phoma</u> spp.	34	21	9
<u>Candida krusei</u>	31	12	5
<u>Gliocladium roseum</u>	30	21	8
<u>Mucor hiemalis</u>	27	16	8
<u>Aspergillus fumigatus</u>	26	19	7
<u>Cladosporium cladosporioides</u>	23	19	9
<u>Geotrichum candidum</u>	18	13	6
<u>Penicillium funiculosum</u>	16	12	8
<u>Rhizopus</u> sp.	16	11	6
<u>Aspergillus versicolor</u>	15	11	7
<u>Rhodotorula</u> spp.	15	13	7
<u>Epicoccum purpurascens</u>	15	11	6
<u>Fusarium roseum</u> ssp. lat.	14	11	7
<u>Aspergillus niger</u>	13	10	7
<u>Aspergillus ustus</u>	13	9	6
<u>Candida lambica</u>	13	11	7
<u>Penicillium corymbiferum</u>	13	11	6
<u>Rhodotorula mucilaginosa</u>	13	11	6
<u>Verticillium lateritium</u>	13	11	5
<u>Aspergillus ochraceus</u>	12	10	6
<u>Penicillium chrysogenum</u>	12	8	3
<u>Fusarium solani</u>	12	9	3
<u>Fusarium</u> spp.	11	9	4
<u>Mucor alternans</u>	11	9	6
<u>Oidiobondron</u> sp.	11	7	6
<u>Penicillium variable</u>	11	9	5
<u>Stemphylium consortiale</u>	10	8	4
<u>Alternaria tenuis</u>	9	7	5
<u>Aspergillus flavus</u>	9	8	6
<u>Rhodotorula glutinis</u>	9	8	5
<u>Stachybotrys atra</u>	9	8	5
<u>Trichoderma alba</u>	9	7	5
<u>Aspergillus terreus</u>	8	7	4
<u>Aureobasidium pullulans</u>	8	6	5
<u>Cunninghamella elegans</u>	8	7	5
<u>Paecilomyces varioti</u>	8	8	4
<u>Penicillium rubrum</u>	8	7	5
<u>Penicillium expansum</u>	7	5	5
<u>Penicillium oxalicum</u>	7	5	3
<u>Trichosporon</u> spp.	7	7	7
<u>Penicillium nigricans</u>	7	7	7
<u>Candida tropicalis</u>	6	3	2
<u>Torulopsis farata</u>	6	5	3
<u>Paecilomyces marquandii</u>	6	4	5
<u>Actinomyces elegans</u>	5	5	4
<u>Aphanomyces</u> sp.	5	4	4
<u>Aspergillus wentii</u>	5	5	4

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<u>Cryptococcus laurentii</u>	5	4	2
<u>Mucor plumbeus</u>	5	4	3
<u>Penicillium commune</u>	5	5	5
<u>Penicillium lanthiellum</u>	5	4	2
<u>Penicillium martensii</u>	5	4	3
<u>Rhodotorula texensis</u>	5	4	3
<u>Torulopsis versatilis</u>	5	3	1
<u>Fusarium aquaeductuum</u>	4	4	4
<u>Monosporium apiosporum</u>	4	3	3
<u>Penicillium implicatum</u>	4	3	3
<u>Pestalotia heterocornis</u>	4	4	4
<u>Pythium spp.</u>	4	4	2
<u>Torulopsis spp.</u>	4	3	2
<u>Penicillium ochrochloron</u>	4	4	3
<u>Myrothecium verrucaria</u>	3	3	2
<u>Penicillium puberulum</u>	3	2	1
<u>Penicillium purpureogenum</u>	3	3	3
<u>Penicillium simplicissimum</u>	3	2	2
<u>Phoma herbarum</u>	3	3	2
<u>Rhizopus chinensis</u> Group	3	3	3
<u>Talaromyces spiculosporus</u>	3	1	1
<u>Absidia glauca</u>	2	2	2
<u>Achlya klebsiana</u>	2	2	1
<u>Allomyces sp.</u>	2	2	2
<u>Aspergillus sp.</u>	2	2	2
<u>Aspergillus flavipes</u>	2	2	2
<u>Beauveria bassiana</u>	2	2	1
<u>Candida spp.</u>	2	2	2
<u>Candida parapsilosis</u>	2	2	2
<u>Cryptococcus luteolus</u>	2	2	2
<u>Curvularia geniculata</u>	2	2	2
<u>Fusarium semitectum</u>	2	2	2
<u>Gliocladium catenulatum</u>	2	2	1
<u>Gliomastix convoluta</u>	2	2	2
<u>Gymnoascus violaceus</u>	2	1	1
<u>Memnoniella echinata</u>	2	2	2
<u>Paecilomyces sp.</u>	2	1	1
<u>Phialophora fastigiata</u>	2	2	2
<u>Talaromyces ucrainicus</u>	2	1	1
<u>Trichosporon cutaneum</u>	2	2	2
<u>Zygorhynchus moelleri</u>	2	2	2

The following species were observed only once in this series of samples:

Achlya sp., Allomyces arbusculus, Ascomycete, Aspergillus chevalieri, A. nidulans, A. sydowii, A. tamarii, A. terreus var. floccosus, Candida curvata, C. intermedia, C. utilis, Cladosporium herbarum, C. sphaerospermum, Cryptococcus diffluens, Curvularia lunata, Doratomyces microsporus, D. stemonitis, Fusarium sambucinum, F. sambucinum var. coeruleum, Gliomastix sp., Helminthosporium sp., Paecilomyces persicinus, Papulospora sp., Penicillium aurantium, P. canescens, P. frequentens, P. javanicum, P. kapuscinskii, P. restrictum Ser., P. sclerotiorum, P. urticae, P. velutinum, P. waksmanii, Phialocephala heterospora, Prototheca sp., Rhodotorula pennaeus, Scopulariopsis brevicaulis, Sepedonium sp., Sporotrichum sp., Stilbella bulbicola, Torula sp., Torulopsis aerea, T. inconspicua, Trichosporon pullulans, Ulocladium atrum, U. tuberculatum, Verticillium sp., Xylaria sp.

be attributed to some degree, however, to the presence of larger amounts of added organic matter or polluting substances.

The list in Table 21 may be presented in several ways. The yeasts that were isolated and identified are listed in Table 23; while yeasts occurred at every station, no single species has yet been found at all nine stations in this system. Without more sampling, it would be difficult to try to explain this. Some of the yeasts listed in Table 23 are known to be associated with human health problems, but usually these problems are restricted to tropical regions. In general, these yeasts parallel those found in other streams that have been contaminated with sanitary sewage.

Such species of fungi as Phialophora jeanselmei, Aspergillus fumigatus, Geotrichum candidum and Monosporium apiospermum (Allescheria boydii) are associated to greater or lesser extent with human disease. In the tropics, Phialophora jeanselmei causes a mycetoma in man but it is not known to do this in temperate regions. Allescheria boydii is an etiological agent of Madura foot. Usually this disease is not contracted unless infested soil reaches the bone tissue of the lower extremities through a deep wound. Aspergillus fumigatus and Geotrichum candidum may cause lung disorders similar to pneumonia if large enough quantities of spores capable of causing aspergillosis or geotrichosis are inhaled. Few, if any, cases of disease are known to have been produced by these fungi in the region of the Cache la Poudre River basin. Rhodotorula species, species of other yeast genera, and Aureobasidium pullulans as well as other fungi, may on occasion enter wounds and cause a secondary infection

or irritation, but these fungi are widespread and the same situation may be true for the populations of the cleanest water or soil areas.

A possibly more important group of organisms in this list may be considered. These cause the disease of plants that grow in the soil and may contact the stream waters especially through irrigation. If spores of fungi capable of causing disease in plants are present in irrigation water they may be added to those already present in the soil and thus increase the chance of producing a less healthy crop. In some, if not all, cases, species of fungi capable of causing disease in plants may be represented by strains that can produce severe disease problems; other strains cannot produce disease. Some of the species listed in Table 21 that can cause plant diseases include: Fusarium oxysporum, F. solani, and F. roseum, strains of all of which are capable of causing vascular wilt diseases or leaf spots on a variety of crop plants; a species of Cephalosporium, possibly represented here, which can cause a severe disease of grasses and grains; species of Rhizopus, Penicillium, Aspergillus, Aureobasidium and other fungi listed that can cause severe problems in the storage and shipment of various kinds of produce; Alternaria tenuis, species of Stemphylium and Curvularia, and species of Phoma, which cause mild to severe infection of leaves and roots of some crop plants, or which attack only the senescent leaves. Strains of other fungi in the list can cause damage of one sort or another to crop plants.

Discussion

On the basis of preliminary, rather casual sampling of waters, river bed materials, and river bank soils, we found that in Colorado, as elsewhere,

relatively large numbers of viable fungus cells can be recovered with the use of nutrient solutions and agars. Among the fungi recovered are species that can cause disease in man if conditions are favorable, and species that can cause disease in the plants of man's crops especially if pathogenic strains are present. Many of these organisms are present in our environment, as shown by their occurrence in locations not contaminated, or presumably not contaminated, by man's activities. When organic matter in the form of various wastes is added to these sites, these organisms use these wastes as food materials and their populations increase, resulting in larger inocula for downstream areas. When waters containing such spores are added to fields with susceptible crops, a potential hazard is created.

At present, to what extent the organic enrichment of the stream or adjacent soils causes an increase in potentially pathogenic fungus populations is not known. Here, reference has been made to organisms pathogenic for man, his livestock, and his crops, or ornamental plants. As organic pollution increases, however, we may expect that some change will occur in the balance set up by nature that holds some of these organisms in check.

E. Berry Creek

Berry Creek is a small permanent stream flowing eastward to the Willamette River from its source on the eastern slopes of the Coast Range in Benton County, Oregon. It originates in the unpopulated and relatively undisturbed lands used by the Department of Forestry, Oregon State University, as an experimental forest.

On September 4, 1961, Harry Phinney of the Department of Botany and Plant Pathology, Oregon State University, collected a series of samples of waters and soils from this creek and airmailed them to Cincinnati where, in the Fungus Studies Laboratory, they were processed for fungal populations by pour plate and shaken flask techniques. In this way, 88 species of filamentous fungi and yeast were recovered (Table 25).

Samples were taken in five areas along the creek, in part related to a pollution study being carried out in an experimental area along it. Below a weir used to determine rate and amount of flow, one reach has been left shaded and unpolluted, then there is a reach from which streamside vegetation has been removed to determine the effect of light on an unpolluted portion of the stream. Below that a portion of the stream is polluted by the regular addition of glucose, the shading plants being left in place, and finally shading plants have been removed. Studied samples were taken from each of these four areas as well as an undisturbed area a quarter mile above the flow-metering weir.

In comparing distribution of fungi in these five sampled areas, at the four types of sites chosen for sampling along a stream, flowing water, water and sediment in pools, water and sediment in riffles, and always irrigated bank soils, it was noted that the usual patterns of distribution were present. Several species appeared commonly in all stations at most sampling sites, and some species were picked up only once or twice, or in only one or two stations. However, there appeared to be no significant difference between the undisturbed area, the shaded areas, and the areas to which glucose had been added whether sunlight was made available or not.

Table 25. Fungi observed in isolations from Berry Creek,
Benton Co., Oregon

Species	Observation Number	Number of Stations (of 25)
Unidentified	150	24
White yeasts	55	15
<u>Penicillium</u> spp.	33	15
<u>Candida guilliermondii</u>	29	16
<u>Phialophora jeanselmei</u>	27	21
<u>Penicillium lilacinum</u>	25	19
<u>Cladosporium cladosporioides</u>	16	16
<u>Fusarium</u> spp.	15	10
<u>Trichoderma viride</u>	15	15
<u>Aureobasidium pullulans</u>	13	13
<u>Candida</u> spp.	13	10
<u>Mucor alternans</u>	12	6
<u>Candida pulcherrima</u>	9	4
<u>Mucor hiemalis</u>	8	3
<u>Candida parapsilosis</u>	7	6
<u>Fusarium aquaeductum</u>	7	7
<u>Penicillium velutinum</u>	6	5
<u>Penicillium janthinellum</u>	5	4
<u>Achlya</u> sp.	4	4
<u>Aphanomyces laevis</u>	4	4
<u>Aspergillus flavus</u>	4	4
<u>Aspergillus versicolor</u>	4	4
<u>Penicillium herquei</u>	4	2
<u>Penicillium ochrochloron</u>	4	4
<u>Torulopsis candida</u>	4	3
<u>Torulopsis lanata</u>	4	3
<u>Alternaria tenuis</u>	3	3
<u>Aspergillus niger</u>	3	2
<u>Aspergillus ustus</u>	3	2
<u>Mucor</u> spp.	3	2
<u>Mucor subtilissimus</u>	3	2
<u>Penicillium gladiolii</u>	3	2
<u>Penicillium restrictum</u> Ser.	3	3
<u>Rhodotorula glutinis</u>	3	2
<u>Rhodotorula mucilaginosa</u>	3	2
<u>Torulopsis datilla</u>	3	3
<u>Chloridium chlamydosporum</u>	2	2
<u>Dictyuchus</u> sp.	2	2
<u>Epicoccum purpurascens</u>	2	2
<u>Geotrichum candidum</u>	2	2
<u>Gliocladium roseum</u>	2	2
<u>Penicillium decumbens</u> Ser.	2	2
<u>Penicillium diversum</u>	2	2
<u>Penicillium nigricans</u>	2	2
<u>Penicillium purpureogenum</u>	2	2
<u>Penicillium thomii</u>	2	2
<u>Phoma</u> spp.	2	2
<u>Rhizopus arrhizus</u>	2	1
<u>Saprolegnia</u> sp.	2	2

The following species were observed only once in this series of samples:

Achlya prolifera, Aspergillus spp., A. clavatus, A. ?melleus, A. ochraceus,
A. tamaritii, A. wentii, Candida ?melinii, C. utilis, Cladosporium avellaneum,
Cunninghamella elegans, Debaryomyces nicotianiae, Gliocladium sp., G. penicilloides,
Gliomastix convoluta, Mucor dispersus, M. fragilis, M. plumbeus, Penicillium
chrysogenum, P. clavigerum, P. fellutanum, P. frequentens, P. funiculosum,
P. lanatum Ser., P. martensii, P. meleagrinum, P. ochrochloron, P. oxalicum,
P. piscarium, P. steckii, P. thomii, P. variabile, P. waksmanii, Phycomycete,
Pichia fermentans, Phoma spp., Rhinocladiella atrovirens, Rhodotorula sp.,
Saccharomyces cerevisiae, Schwanniomyces alluvius, Scopulariopsis brevicaulis,
Stemphylium sp., Trichosporon cutaneum.

In order to present more meaningful information about fungal populations of natural streams modified for various types of study, it is necessary to have information from a number of sampling dates, and additional types of fungus isolation procedures, preferably on a year-round sampling basis during at least one year when types of pollution are correlated in such a way that the information about fungus populations in relation to pollution can be determined without reference to competing problems.

F. Duck Creek

Duck Creek is a small, originally intermittent, stream which originates in the City of Cincinnati, Hamilton County, Ohio, on the north facing slope of the hills formed by the Norwood Trough, and flows in a generally northward and eastward direction into the Little Miami River. Part of the flow is in the City of Norwood, and part in Hamilton County. Most of the flow is through densely populated areas and industrial areas, and part of this lies in underground storm sewers. Samples of water, stream bed materials and bank soils were obtained by Dr. Rinji Kuwahara, Hokkaido, Japan, and the writer in September, 1961. These were obtained in an industrially polluted reach of the stream in a period of low flow when there was no obvious pollution by industry. Pour plates and shaken flask cultures were made in the Fungus Studies Laboratory as a demonstration for Dr. Kuwahara of techniques which can be used in the study of such populations, and of the populations which can be recovered by those techniques.

No data are available concerning the stream flow or pollution loading of this stream, so that at present, with only one set of samples studied, no correlations can be made between the 42 species of fungi recovered (Table 26), and the type of pollution, season of occurrence, or permanence of residence of the fungi found.

G. Columbia River

In 1955, J. N. Wilson sent three samples of slimes, thought to be largely of Sphaerotilus natans, to Cincinnati for processing in the Fungus Studies Laboratory. Through the courtesy of Dr. A. F. Bartsch, on January 2, 1962, eight samples of Columbia River water were obtained and shipped to the Fungus Studies Laboratory for study. The samples of slimes were obtained from the river bottom from stones near the outfall of a factory yielding paper mill wastes. Both sets of samples were processed by using pour plates, the second set, in addition, was processed in shaker flasks using five dilutions of the sample in two media as described in the technique for the enumeration of yeasts. Fourteen species of fungi (Table 27) were obtained from the slime samples, 23 species of filamentous fungi and yeasts and yeast-like fungi from the water samples, yielding a total of 27 species of fungi obtained to date from Columbia River samples.

The slime sample showed that in addition to Sphaerotilus natans, which was present, there were many fungi forming slimes in the river bed on stones, emplacements of various sorts, and other obstructions, which were probably feeding on dissolved, colloidal and even solid organic

Table 26. Fungi observed in isolations from Duck Creek,
Hamilton Co., Ohio

Species	Observation Number	Number of Stations (of 10)
Unidentified	78	10
<u>Candida parapsilosis</u>	17	8
<u>Pichia fermentans</u>	13	9
<u>Penicillium</u> spp.	12	6
<u>Phoma</u> spp.	11	5
<u>Cladosporium</u> sp.	8	5
<u>Cladosporium cladosporioides</u>	6	5
<u>Hansenula</u> sp.	5	3
<u>Rhodotorula glutinis</u>	4	4
<u>Alternaria tenuis</u>	3	3
<u>Fusarium oxysporum</u>	3	2
<u>Trichoderma virida</u>	3	3
<u>Torulopsis farata</u>	3	3
<u>Aphanomyces</u> sp.	2	2
<u>Candida curvata</u>	2	2
<u>Candida krusei</u>	2	2
<u>Geotrichum candidum</u>	2	2
<u>Gliocladium</u> sp.	2	2
<u>Penicillium lilacinum</u>	3	3
<u>Phycomycete</u>	2	2
<u>Rhodotorula</u> spp.	2	2
<u>Trichosporon cutaneum</u>	2	2
<u>Cephalosporium</u> sp.	2	2

The following species were observed only once in this series of samples:

Achlya sp., Aspergillus fumigatus, A. sydowii, Aureobasidium pullulans, Candida sp., Coniothyrium sp., Fusarium sp., Fusarium solani, Gliocladium roseum, Libertella sp., Monochaetia kansanensis, Mucor sp., M. hiemalis, Myrothecium verrucaria, Penicillium chrysogenum, P. commune, P. herquei, P. ochrocloron, P. sclerotiorum, Phialophora jeanselmei.

Table 27. Fungi observed in isolations from miscellaneous streams*

Species	Observation Number
Unidentified fungi	121
<u>Penicillium</u> spp.	64
<u>White yeasts</u>	53
<u>Moniliaceae</u>	52
<u>Geotrichum candidum</u>	40
<u>Rhodotorula</u> spp.	40
<u>Trichoderma viride</u>	40
<u>Fusarium</u> spp.	31
<u>Phialophora jeanselmei</u>	30
<u>Penicillium lilacinum</u>	26
<u>Aspergillus fumigatus</u>	25
<u>Cladosporium cladosporioides</u>	22
<u>Fusarium aquaeductuum</u>	22
<u>Penicillium funiculosum</u>	20
<u>Alternaria tenuis</u>	16
<u>Fusarium oxysporum</u>	12
<u>Mucor fragilis</u>	12
<u>Penicillium ochrochloron</u>	12
<u>Aspergillus niger</u>	10
<u>Aspergillus versicolor</u>	10
<u>Phoma</u> spp.	11
<u>Cephalosporium</u> spp.	8
<u>Mucor</u> spp.	8
<u>Paecilomyces varioti</u>	8
<u>Aspergillus flavus</u>	7
<u>Aspergillus ochraceus</u>	6
<u>Aureobasidium pullulans</u>	6
<u>Epicoccum purpurascens</u>	6
<u>Dematiaceae</u>	5
<u>Mucor plumbeus</u>	4
<u>Myrothecium verrucaria</u>	4
<u>Penicillium chrysogenum</u>	4
<u>Penicillium lanthiellum</u>	4
<u>Penicillium oxalicum</u>	4
<u>Ulocladium tuberculatum</u>	4
<u>Aspergillus candidus</u>	3
<u>Aspergillus terreus</u>	3
<u>Dictyuchus monosporus</u>	3
<u>Penicillium commune</u>	3
<u>Saprolegnia ferax</u>	3
<u>Achlya americana</u>	2
<u>Aspergillus</u> spp.	2
<u>Aspergillus clavatus</u>	2
<u>Aspergillus ustus</u>	2
<u>Candida humicola</u>	2
<u>Coniothyrium</u> sp.	2
<u>Gliocladium roseum</u>	2
<u>Penicillium herquei</u>	2
<u>Penicillium luteum</u>	2
<u>Penicillium nigricans</u>	2
<u>Penicillium variable</u>	2
<u>Penicillium viridicatum</u>	2
<u>Rhizopus</u> sp.	2
<u>Rhizopus arrhizus</u>	2
<u>Rhizopus rhizopodiformis</u>	2
<u>Torulopsis lamata</u>	2
<u>Trichosporon capitatum</u>	2
<u>Trichosporon cutaneum</u>	2
<u>Zygorhynchus moelleri</u>	2

The following species were observed only once in this series of samples:

Achlya diffusus, Aspergillus carneus, A. sydowii, A. ustus var. laevis, Candida pulcherrima, Cladosporium sphaerospermum, Gliocladium catenulatum, Gliomastix convoluta, Humicola grisea, Leptomitius lacteus, Mucor corticolous, M. hiemalis, Paecilomyces sp., Penicillium cyclopium, P. expansum, P. granulatum, P. rubrum, P. stipitatum, Phoma herbarum, Pyrenochaeta sp., Pythium sp., Rhodotorula mucilaginosa, R. texensis, Saccharomyces sp., Stemphylium botryosum, Stilbellaceae, Trichoderma alba, Verticillium sp., V. lateritium.

* Streams from which samples were obtained:

Lawrenceburg, Indiana. Tanners' Creek. Antibiotics wastes 3 sets
Ohio River, Cincinnati, Ohio 3 samples
Ottawa River, Lima, Ohio - Oil Refinery and municipal wastes. 4 samples
Fort Ancient Creek, Warren Co., Ohio - 4 samples
Cowan Creek, Clinton Co., Ohio - 4 samples
East Fork, of the Little Miami River, Batavia, Clermont Co., Ohio - aquatic fungi
Great Miami River, Hamilton, Ohio - 4 samples
Columbia River, Oregon (near Portland) - 3 samples slime on rocks 1955
Columbia River, Oregon (near Portland) - 8 water samples, 1962
Laurel, Alabama, stream carrying municipal wastes - 4 samples
San Marcos Dam, California - 1 sample of slime.

matter being carried by the river. The water samples showed that a number of species of filamentous fungi and yeasts were present in the river water, the yeasts numbering between more than 100 and 100,000 cells per milliliter of water. The presence of such populations indicates that supplies of nutrients are available to these microorganisms as organic pollutants regardless of the source of pollution, and that these organisms are able to survive and grow in the presence of substances thought to be potentially toxic.

H. Tanner's Run (Tributary)

Tanner's Run is a small stream which flows into the Ohio River at Lawrenceburg, Dearborn County, Indiana. It passes several distilleries and other industries. In 1952-1953 one of the distilleries produced antibiotics and other pharmaceuticals, a field of manufacture which was dropped shortly afterward. The waste products of penicillin and streptomycin manufacture were discharged directly to Tanner's Run through a small tributary. Three sets of samples were made through the courtesy of the plant engineer from this creek. These were processed in the Fungus Studies Laboratory at Cincinnati. Only pour plates were prepared of these samples but 28 species of fungi were recovered. The creek usually carried materials yielding a heavy scent of amyl acetate.

As the plant effluent left a drain in a trash dump at the edge of a paved parking lot, its spray washed tree trimmings, twigs, branches, lumber, etc., and the water flowed over similar materials. In all materials washed by this effluent a grey to pink colored slime developed and this also occurred on the soil bank of the creek wherever the water washed the

bank. At one of the times when samples were obtained a pool had developed in the creek which was covered on the bottom with apparently coarse pebbles of white sand which turned out to be viable mycelial balls of Penicillium chrysogenum. It is suggested that the strain of Streptomyces griseus used in the production of streptomycin had been patented and thus had to be destroyed before being released as a waste after a streptomycin production run, but the Penicillium chrysogenum strain for the production of penicillin, being public property, could be released without the expensive killing process. While most of the hillside down which this effluent creek ran was steep, allowing for the rapid run of waste water, at one point there was a bench which slowed the water down enough so that in the woods on either side of the creek there became built up a large deposit of soft sludge which dried out slowly. The nature of this sludge was not determined, but it yielded a number of fungi.

From a study of materials such as this, on the limited basis on which it was carried out, it appears that untreated organic industrial waste materials contain sufficient nutrients to support a relatively large population of fungi which serve as a form of natural treatment process for such wastes.

I. Miscellaneous

In the process of sampling Lytle Creek stations, additional creeks were hunted in which it could be thought the water was clean and could be used as checks. Two such creeks were sampled. One was Cowan's Creek (28 species) some distance below the dam which forms Cowan's Lake. Here

there seemed to be a minimum of human pollution sources, only a few farms occurring in the area and the homes on these being set some distance from the creek. There was also a minimum of animal pollution at the sampled point. The second was a small intermittent stream (26 species) which drained the valley south of the hill on which Fort Ancient is located. The single farmhouse in this drainage lies at the head of the valley and no surface drainage from it ever reaches the sampled point. At neither point were fungus populations (43 species, combined) significantly different from those found at the upper stations on Lytle Creek.

A set of water samples from the Ohio River, taken in the spring of 1952 in connection with the water storage of Histoplasma capsulatum spores project, was processed. A total of 25 species was recovered, mostly of fungi which were similar to those found in Lytle Creek and elsewhere.

Twenty-two species of fungi were recovered from slimes collected from stones in the Ottawa River, near Lima, Allen County, Ohio, in the winter of 1956, four and seven miles below the outlet of an oil refinery.

At several points above and below a paper mill at Hamilton, Butler County, Ohio, samples of water, bottom materials and bank soils were taken on the Great Miami River. These yielded 36 species of fungi some of which are known to be cellulolytic.

Samples of mud from Lafayette, Alabama, from a stream carrying sanitary sewage, yielded 18 species of fungi including some of the commoner species associated with municipal wastes.

In all these cases of "one-shot" sampling, where one or several samples were taken at one to several points on a stream, but at only one time, it can only be said that there seems to be a definite series of fungi associated with polluted or organically enriched waters, that many of these species occur in the cleaner reaches of a stream, that as the pollution load increases the numbers of colonies increase at least of those more readily adaptable to the organic enrichment, that these fungi are present regardless of the type of pollution involved, and that at least some of these species form part of a normal biota of streams whether polluted or not.

V. Fungi in Streams Carrying Acid Mine-Drainage*

Introduction

In the study of problems arising from the pollution of streams by acid mine-drainage, little attention has been paid to the fungi and the possible relation of such organisms to various factors involved in this type of pollution. The extensive annotated review of the literature by McFadden in Moulton (1957) indicates that, although problems dealing with acid mine-drainage were recognized as early as 1902, at least in the United States, with few exceptions no one had reported on the occurrence and activity or even the presence of fungi in such situations. Lackey (1938, 1939) reported on the flora and fauna of waters associated with coal mines, especially in streams and pools receiving drainage from such mines, but only in the second paper, in which he mentioned the observation of two unidentified species of fungi, did he recognize that fungi occurred in such habitats.

Ruchhoft, Ettinger and Walker (1940), in studying self-purification of natural waters in the Ohio River, concluded that "the biological self-purification reaction that occurs in acid sewage at pH values of 2.0 to 3.0 is largely the result of biochemical oxidation which is being carried on by a limited number of biological species." The authors identified these species only as "not bacterial."

Two types of bacterial activity have been studied intensively in connection with the interpretation of the acid mine-drainage problem. The first type, and probably the more important, is centered about the species of bacteria associated with the formation of ferrous sulphate,

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sulphuric acid, and ferric hydroxide from iron sulphides in the naturally occurring minerals associated with coals of all types from lignite to anthracite. Moulton (1957), Fjerdingsstad (1958), Brant and Moulton (1960), and the House Committee Report (1962), among others, summarize the results of these studies. The second is centered around the fate of bacterial indicators of pollution in highly acid waters. Some of this work has been referred to by McFadden in his annotated bibliography (Moulton, 1957) and by Wilson, Hipke, and Rogers (1964).

In a preliminary study reported to the Purdue Industrial Waste Conference in 1965, Wilson, Hipke, and Rogers (1965) found "at pH levels 5.5 and above, sewage stabilization will be the result primarily of bacterial activity. Below pH 5.0, and particularly pH 4.5 and lower, the predominating microflora, probably yeasts, will determine the degree of stabilization." Dr. Wilson and his students were working with raw sewage and polluted water from the Monongahela River containing both acid mine-drainage, municipal sewage, and other wastes.

Because fungi had been shown to be associated with natural streams as well as streams that had been modified with various kinds of polluting substances (Cooke, 1957a, 1960), it was suggested that a survey of fungi in streams carrying acid mine-drainage be carried out (in cooperation with two of the field laboratories investigating acid mine-drainage) to determine the role of fungi in such habitats. Even though the project was discontinued before this latter step could be investigated, certain data related to the survey will be presented here.

Materials and Methods

For sampling purposes, three principal areas were chosen for study. The first was the Brush Creek-Hocking River valley area in southeastern Ohio. Samples could be collected readily in the field and brought to the refrigerator the same day or on the following afternoon. The second area was the Monongahela River basin near Morgantown, West Virginia. Here, after our personnel had made a preliminary sampling trip in the area, personnel from the Wheeling Laboratory of the Ohio River Basin Project, FWPCA, could collect samples and send them the following day to Cincinnati by bus express. The third was the Elkins Demonstration Project area on Roaring Creek and Grass Run tributaries of the Tygart River, itself a tributary of the Monongahela River. Personnel at the Elkins Project station carried out sampling after the first visit to each of the stations chosen by the project leader. The Elkins area, also in West Virginia, is separated from the Morgantown area by about 90 air miles, and the area in Ohio is about 180 air miles from the Morgantown area. All streams sampled drain into the Ohio River.

Stations chosen for sampling usually lay on apparently unpolluted, apparently mildly polluted, and apparently heavily polluted portions of the stream. An attempt was made to include stations at which sanitary sewage was also being received by the stream. Sampling containers were 16 dram or 60 ml plastic vials with snap cap closures. At each station grab samples were obtained from the running stream, from sediment from the stream bottom, from mud from the creek bank at the interface of the

water and the soil, and from soil at the top of the creek bank that was apparently flooded only by the more severe floods. The four types of samples were numbered appropriately and packed in a container for transport to Cincinnati. Upon arrival at the Fungus Studies Laboratory, samples were refrigerated overnight or until opportunity was available for working with them. Samples were obtained at the same stations, in the same ways, in spring, autumn and winter periods. Chemical determinations were made of the supernatant of water samples and of a 1:10 dilution of the other samples; pH was determined on a glass electrode pH meter; iron determinations were made by Henry Tabak using a modification of techniques described by Harvey, Smart, and Ames (1955); total organic carbon analyses were made by Mr. R. T. Williams and his staff with a Beckman Carbon Analyzer.

Most of the techniques used in studying the samples have been described above (Chapter II). Parallel sets of plates and cultures were prepared with these media: hemp seed baits for true aquatic fungi, pour plates using neopeptone - dextrose - Aureomycin agar with and without rose bengal; and Yeast Nitrogen Base - 1% and 20% glucose shaken flasks with 1:10 dilutions of the original samples.

After proper periods of incubation on the laboratory bench at room conditions of light and temperature, or on the shaker, shaker flask cultures were streaked on malt extract - yeast extract - glucose agar plates for development of yeast colonies, and mold growths from such flasks were planted on similar agar. Agar plates were counted and species inventoried and picked for later identification; hemp seeds on which aquatic fungi developed were

transferred to fresh hemp seed plates. At least 200 species or species groups of fungi were identified from the resulting colonies. "Species groups" is used to include groups of cultures usually identifiable to genus but not to species, single species for which no readily available name could be obtained, and groups of cultures not producing spores or identifiable fruiting structures.

Results

In the spring of 1954 several samples of water, bottom sediments, and slime growths appearing on stones in streams carrying "yellow boy" in Athens and Perry Counties in southeastern Ohio, had been tested for the occurrence of fungi. They were found to have at least 23 species that were common in other streams that had been tested at that time. In the autumn of 1963 a series of water samples was returned from the Monongahela River basin. Portions of these samples yielded a total of 24 species or species groups of fungi, also representing relatively familiar species. Against this background of information, the larger surveys could be developed, at least in part. Since samples from these two sets of material were not obtained at the same points as those sampled in the larger principal survey, they will not be considered further; however, the master list of 202 species and species groups includes 6 obtained only in these two sets of samples.

Sources of the samples tested are given in Tables 28a, 29a, and 30a, for the three areas in which samples were studied. In Tables 28b, 29b, and 30b, data are presented on the pH of each sample, the total iron, and the

Table 28a. Habitat Data, Ohio Series

Station	Location
1	Sandy Run at King Hollow Bridge, off SR 278, Vinton Co., Ohio
2	Sandy Run below mine at mouth of Coal Hollow, along SR 278, Vinton Co., Ohio
3	Sandy Run above mine at mouth of Coal Hollow, Vinton Co., Ohio
4	Sunday Creek at Junction of Perry Co. Route 291 and SR 13, Perry Co., Ohio
5	Sunday Creek near Hemlock above bridge on SR 165, Perry Co., Ohio
6	Spring (bricked in) on hillside berm of SR 216 near Murray City, Hocking Co., Ohio
7	Creek from spring in ochre "cave," east of New Straitsville, along SR 216, Hocking Co., Ohio ^a
8	Crumley's Farm creek, off US 33 near Groveport, Franklin Co., Ohio ^b
9	Ash Cave Falls, Ash Cave State Park, Hocking Co., Ohio ^b

^a - No apparent mining activity but a heavy deposit of "yellow boy."

^b - Selected as clean water control stations.

Table 28b. pH and Chemical Data, Ohio Series

Station	Habitat	pH			Total Fe ppm 2-22-65	Fe ⁺⁺ in presence of Fe ⁺⁺⁺ ppm 2-22-65
		3-17-64	10-11-64	2-22-65		
1	1 W ^a	6.2	3.8	6.5	113	20
	2 S	6.4	4.0	4.2	600	10
	3 B	6.2	4.0	---	---	--
	4 U	5.1	3.5	5.6	1,080	20
2	5 W	3.2	3.8	6.5	1,480	780
	6 S	4.8	3.7	3.3	6,250	10
3	7 W	6.5	---	5.5	15	0
	8 S	6.7	---	5.6	300	2
4	9 W	3.3	4.2	(6.7) ^b	680	420
	10 S	3.9	4.7	(4.5)	700	15
	11 B	4.4	3.8	(5.1)	12,000	280
	12 U	3.8	7.2	(6.7)	3,050	80
5	13 W	3.2	3.8	4.0	500	200
	14 S	3.6	3.8	3.1	4,150	15
	15 B	3.4	4.7	3.1	4,250	18
	16 U	4.1	6.5	5.0	2,050	20
6	25 W	3.2	4.6	2.0	720	360
	26 S	3.2	3.7	3.0	1,700	380
7	21 W	3.5	4.5	5.3	800	0
	22 S	3.6	4.0	3.0	12,000	60
	23 B	2.9	3.7	3.0	12,000	37
	24 U	4.2	6.7	4.2	770	3
8	27 W	7.1	7.2	---	---	---
	28 S	7.1	7.4	---	---	---
	29 B	7.2	7.3	---	---	---
	30 U	7.4	7.3	---	---	---
9	29 W	---	6.4	5.3	0	0
	30 S	---	6.4	5.4	2	0
	31 B	---	6.4	---	---	---
	32 U	---	6.4	---	---	---

NOTE: ^aW Water
 S Sediment
 B Bank Soil
 U Upland Soil
 --- Not Sampled

^bFigures from station 0.2 mi.
 down stream.

Table 28c. Colonies and Species Per Gram Sample

Station	Habitat	3-17-64		10-11-64		2-22-65	
		Colonies	Species	Colonies	Species	Colonies	Species
1	1 W	12,000	9	4,300	10	8,400	5
	2 S	68,000	18	4,300	18	6,500	15
	3 B	570,000	15	6,400	17	-----	--
	4 U	58,000	17	10,400	15	58,000	13
2	5 W	150,000	9	3,700	6	9,000	6
	6 S	82,000	17	4,900	14	4,600	15
3	7 W	12,000	8	-----	--	12,000	10
	8 S	68,000	11	-----	--	2,300	10
4	9 W	12,000	6	480	4	72,000	4
	10 S	68,000	18	8,200	18	3,000	12
	11 B	86,000	25	9,400	20	6,600	7
	12 U	29,000	15	25,000	22	38,000	13
5	13 W	9,000	5	480	6	6,600	6
	14 S	5,400	16	3,600	11	8,700	19
	15 B	5,700	20	120,000	15	12,000	16
	16 U	38,000	14	31,000	14	61,000	15
6	25 W	45,000	7	720	6	9,000	5
	26 S	13,000	9	17,000	17	1,900	4
7	21 W	9,600	3	240	4	8,400	7
	22 S	4,600	17	1,400	12	13,000	11
	23 B	75,000	10	8,700	19	30,000	9
	24 U	61,000	10	54,000	17	580,000	13
8	27 W	11,000	8	3,700	13	-----	--
	28 S	4,300	19	920	10	-----	--
	29 B	72,000	20	42,000	17	-----	--
	30 U	38,000	22	37,000	18	-----	--
9	29 W	-----	--	1,100	11	7,800	7
	30 S	-----	--	3,500	15	2,600	9
	31 B	-----	--	15,000	19	-----	--
	32 U	-----	--	3,600	12	-----	--

Table 29a. Habitat Data, Morgantown Series

Station	Location
1	50 yd. above mouth of Scott's Run, off W. Va. Rte. 100
2	Decker's Creek below power plant, about 1 mi. above mouth, Morgantown
3	Booth Creek, 2.1 mi. above Weirton Steel Co. coal mine preparation plant ^a
4	Booth Creek at Weirton Steel Co. coal mine preparation plant between gob piles and settling basin (abandoned)
5	Lake Lynn on Cheat River at boat docks below State Park Lodge
6	Near mouth of Robinson Run, 1.7 mi. N of US 19 on right of road
7	Monongahela River, west bank, 2.5 mi. N of US 19 bridge below Morgantown
8	Monongahela River, east bank, 0.5 mi. S. of Morgantown Dam
9	Wheeling Creek, Blaine Co., Ohio, just above Belmont Co. road #10 bridge below active coal mine

^aSelected as a clean water control station.

Table 29b. pH and Chemical Data

Station	Habitat	pH			Total Fe ppm	Fe ⁺⁺ in presence of Fe ⁺⁺⁺		Fe ⁺⁺ in presence of Fe ⁺⁺⁺	Fe ⁺⁺ in presence of Fe ⁺⁺⁺
		5-22-64	11-6-64	2-16-65		11-6-64	2-16-65	11-6-64	2-16-65
1	1 W	4.4	4.0	4.8	290.0		70.0	2.5	0.6
	2 S	4.0	4.0	3.9			350		20
	3 B	4.2	4.1	4.2			2,850		30
	4 U	4.0	4.4	5.0			2,100		100
2	5 W	4.9	5.4	5.5	0		10	0	0.7
	6 S	7.1	5.4	6.4			1,000		40
	7 B	7.1	7.1	6.8			800		30
	8 U	7.6	7.2	6.9			500		20
3	9 W	6.8	7.2	6.8	0		1.2	0	0
	10 S	6.3	6.9	5.5			500		20
	11 B	6.2	6.3	6.9			140		10
	12 U	5.6	5.2	6.6			300		17.5
4	13 W	5.0	4.5	4.4	2.5		5.4	0.1	0
	14 S	4.3	4.0	3.9			680		20
	15 B	4.5	4.2	3.9			570		26
	16 U	2.9	3.8	3.4			700		20
5	17 W	5.5	5.4	4.6	0		0.87	0	0
	18 S	7.0	6.6	5.2			1,800		106
6	19 W	3.0	3.8	3.3	630		197	130	1.87
	20 S	3.0	3.7	3.0			5,300		14
7	21 W	6.0	5.1	6.3	0.3		3.6	0.1	1.88
	22 S	4.6	5.8	5.8			3,300		413
	23 B	6.4	5.5	5.4			400		8
	24 U	6.0	5.1	5.7			500		0
8	25 W	5.2	5.6	5.5	0.6		2.32	0.2	2.32
	26 S	6.0	6.2	6.6			4,500		3,200
	27 B	6.4	5.5	6.1			180		4
	28 U	5.8	5.6	6.1			530		10
9	29 W	---	6.3	6.4	10.0		17.4	0.6	0
	30 S	---	6.8	6.6			980		330
	31 B	---	6.8	2.7			5,200		76
	32 U	---	3.5	2.9			1,000		16

Table 29c. Colonies and Species Per Gram Sample

Station	Habitat	5-22-64		11-6-64		2-16-65	
		Colonies	Species	Colonies	Species	Colonies	Species
1	1 W	12,000	13	51,000	16	81,000	10
	2 S	13,000	16	19,000	22	3,000	14
	3 B	570,000	22	36,000	14	109,000	26
	4 U	110,000	22	43,000	16	118,000	19
2	5 W	20,000	11	270	4	8,200	7
	6 S	220,000	21	2,300	14	18,000	12
	7 B	470,000	21	24,000	16	25,000	12
	8 U	11,000	15	2,900	16	68,000	12
3	9 W	36,000	8	2,400	6	2,600	3
	10 S	62,000	17	5,400	15	36,000	14
	11 B	11,000	18	4,100	8	11,000	14
	12 U	160,000	13	26,000	15	8,600	12
4	13 W	7,400	7	5,300	9	630	4
	14 S	30,000	24	37,000	9	11,000	16
	15 B	35,000	21	4,200	11	32,000	14
	16 U	3,300	6	1,000	8	4,000	13
5	17 W	6,800	6	1,600	4	180	1
	18 S	16,000	20	1,400	16	4,900	15
6	19 W	1,700	5	3,200	8	410	4
	20 S	16,000	17	5,900	19	3,100	12
7	21 W	70,000	6	630	8	1,500	5
	22 S	120,000	20	850	15	5,300	11
	23 B	102,000	22	---	6	13,000	17
	24 U	89,000	14	58,000	21	38,000	8
8	25 W	5,700	8	1,800	4	1,800	3
	26 S	6,100	15	1,000	16	68,000	12
	27 B	62,000	10	4,100	18	16,000	15
	28 U	1,100,000	14	59,000	17	40,000	11
9	29 W	---	--	4,200	11	5,400	5
	30 S	---	--	8,500	22	5,800	7
	31 B	---	--	4,600	19	2,100	7
	32 U	---	--	8,600	7	17,000	13

Table 30a. Habitat Data, Elkins Series

Station	Location
1	Station R-1, near mouth of Roaring Creek, 100 ft. above bridge.
2	Station G-1, near mouth of Grassy Run at old railroad bridge
3	Station GT-6-1, Norton Mine #15 drainway
4	Station RT5-3, Pond on White's Run, top of strip pit
5	Station R-3, Roaring Creek at Coalton
6	Station TR6-17, Kittel's Run Samples 25-28 include samples of material from aluminum and iron "travertine" deposits built up at springs on leaf litter
7	Station R-C Special (R-9), Roaring Creek ^a
8	RT-6 Basin - Pond in strip pit by Coalton Dump
9	Station RT-10-1. Brook's Run, tributary of Roaring Creek ^b

^aOriginally selected as a clean water control but strip pit opened at or near headwaters.

^bSelected as a clean water control.

Table 30b. pH and Chemical Data

Station	Habitat	pH			Total Fe ppm	Fe ⁺⁺ in presence of Fe ⁺⁺⁺	
		6-11-64	11-6-64	2-26-65		11-6-64	2-26-65
1	1 W	4.3	5.5	5.6	8.80		0
	2 S	3.4	4.0	3.5		7,100	136
	3 B	3.4	4.0	3.2		10,500	540
	4 U	4.1	4.5	3.9		5,150	420
2	5 W	3.3	4.0	3.3	130.0	130	1
	6 S	3.2	3.8	2.9		6,800	836
	7 B	3.1	4.1	3.0		3,860	336
	8 U	3.5	5.0	4.4		2,900	80
3	9 W	3.3	4.0	3.2	220.0	240	0.8
	10 S	3.3	3.8	3.3		3,000	20
	11 B	3.4	3.8	6.0		2,150	20
	12 U	3.3	4.1	3.4		5,400	72
4	13 W	5.9	5.5	5.0	0	0.6	0
	14 S	5.9	5.8	5.2		680	26
	15 B	6.5	5.7	4.8		800	120
	16 U	5.8	6.0	6.4		840	60
5	17 W	7.33	5.0	4.6	0.18	3.0	0
	18 S	5.5	4.9	4.0		3,700	720
	19 B	5.3	5.0	4.2		3,700	130
	20 U	4.7	5.2	4.4		1,300	32
6	21 W	3.1	3.9	3.0	315.0	244	2
	22 S	6.8	3.9	3.1		7,080	80
	23 B	4.5	3.7	6.3		8,500	2,100
	24 U	6.8	4.2	4.5		2,100	36
	25 T*	3.1	4.6	3.0		11,000	140
	26 T	3.0	4.3	3.1		10,500	70
	27 T	5.3	3.8	2.9		11,100	116
	28 T	3.7	3.8	2.9		11,200	130
7	29 W	6.1	5.7	4.4	0	1.25	0
	30 S	5.0	6.3	5.0		200	10
	31 B	5.9	6.1	6.8		150	0
	32 U	7.3	5.3	5.5		850	36
8	33 W	3.3	4.2	3.4	370	244	0.3
	34 S	3.2	4.2	3.5		11,100	610
	35 B	3.2	4.1	3.2		11,000	74
	36 U	3.0	4.0	3.1		7,000	40
9	37 W	5.7	5.6	5.0	0.24	1.0	0
	38 S	6.1	7.2	6.4		100	0
	39 B	6.9	6.6	6.3		800	20
	40 U	6.9	7.0	6.4		800	52

Table 30c. Colonies and Species Per Gram Sample

Station	Habitat	6-11-64		11-6-64		5-26-65	
		Colonies	Species	Colonies	Species	Colonies	Species
1	1 W	110,000	8	3,000	1	730	5
	2 S	25,000	20	4,400	12	29,000	13
	3 B	21,000	13	2,900	15	12,000	15
	4 U	1,200,000	11	45,000	15	12,000	11
2	5 W	19,000	11	1,000	2	2,900	7
	6 S	99,000	11	9,200	18	2,300	15
	7 B	90,000	17	6,400	18	4,300	11
	8 U	63,000	12	23,000	18	98,000	9
3	9 W	1,000	5	2,200	5	960	5
	10 S	1,300	22	3,100	9	2,900	10
	11 B	12,000	16	5,100	16	5,500	14
	12 U	190,000	9	16,000	9	36,000	11
4	13 W	27,000	10	4,900	5	5,000	10
	14 S	12,000	12	2,500	12	2,100	9
	15 B	26,000	19	6,000	18	3,900	12
	16 U	240,000	14	53,000	12	22,000	13
5	17 W	25,000	5	2,500	3	3,100	11
	18 S	71,000	12	3,700	13	4,600	10
	19 B	68,000	8	2,700	16	6,600	8
	20 U	330,000	14	23,000	12	21,000	11
6	21 W	4,900	11	660	4	6,000	9
	22 S	6,800	15	3,700	12	26,000	14
	23 B	35,000	9	14,000	13	19,000	13
	24 U	110,000	19	34,000	11	20,000	9
	25 T	74,000)		280		540	
	26 T			2,100		160	
	27 T			250		600	
	28 T			1,500		2,200	
7	29 W	2,100	7	5,500	6	5,800	8
	30 S	13,000	15	2,600	12	1,500	14
	31 B	13,000	11	3,700	15	2,700	12
	32 U	200,000	9	9,100	7	33,000	16
8	33 W	39,000	6	540	5	4,000	8
	34 S	1,300	8	3,700	17	4,200	11
	35 B	1,000	12	14,000	10	14,000	16
	36 U	56,000	14	200,000	9	13,000	7
9	37 W	130,000	4	85,000	5	1,700	9
	38 S	14,000	12	2,000	18	2,700	9
	39 B	15,000	11	2,900	17	6,300	13
	40 U	90,000	4	14,000	13	20,000	11

ferrous in the presence of ferric iron 17 mg/l. In Tables 28c, 29c, and 30c, are given the numbers of colonies of fungi per gram dry weight of sample as counted on pour plates prepared in neopeptone - dextrose - rose bengal - Aureomycin agar. The numbers of species observed on all media are also listed. Colony counts on acidified neopeptone - dextrose - Aureomycin agar were usually higher than those on other media, at least where the pH of the samples tested was below 5.0.

Of the 196 species of fungi obtained in three seasons of sampling in three areas of soft coal mining, the Brush Creek - Hocking Valley, Ohio, samples yielded 151 or 78% of the total species; the Morgantown area, 131, or 68% of the total; and the Elkins area, 122 or 63% of the total. Stated in a seasonal way, in the spring 123, or 63% of the total were recovered; in the autumn 146, or 75% of the total; and in the winter 108, or 56% of the total. Of the total list, 44 or 23% of the species and species groups representative of yeasts are included. Of the species of all groups, a relatively large number, 10.3%, appeared in all three areas sampled in all three seasons. The following tabulation shows the distribution of species on the basis of numbers of species represented in all or part of the nine sets of samples:

<u>Number of sample sets</u>	<u>Number of species</u>	<u>Percent of total</u>
9	20	10.3
8	8	4.1
7	17	8.8
6	12	6.2
5	10	5.2
4	16	8.3
3	23	11.8
2	30	15.5
1	58	29.8

This distribution is unusual because of the relatively large number of species, 83, or 43.9% of the total, that appear in four or more sets of samples.

An attempt was made to determine the similarity of one type of population with another. The coefficient of similarity formula was applied to data on species listed for the four habitats in each station in each area sampled. There was a relatively high degree of similarity among the species lists - water included 88 species, or 45% of the total lists; sediment, 137 species, or 70%; bank soil, 143 species, or 73%; and upland soil, 133 species, or 68%. This appeared to be true regardless of whether the species list for one of the four habitats was shorter than those for the other three. Also, the species lists for the three areas, the three seasons, all the water, sediment, bank soil and upland soil samples, or any other combination of the samples tested, were relatively similar. On the individual sample level, 30-50% of the species found in one sample was present in the others; on the group level, where several areas or seasons were being compared, 30-60% of the species was held in common by any paired combination.

Discussion

Species of fungi, including aquatic Phycomycetes, yeasts, and filamentous soil molds, have been found in relatively large numbers in waters carrying acid mine-drainage, in stream bottom sediments and bank soils over and around which such waters pass, and on the upper parts of the banks of streams that may be reached by such water in times of heavy

runoff of excess precipitation. The streams carrying acid mine-drainage, associated sediments, and bank soils usually have a low pH and a relatively high or very high amount of iron as precipitation, referred to as "yellow boy", in the shallower parts of the streams and on the shore. Thus, the species of fungi that have been found are subjected to high degrees of acidity and large amounts of iron compounds in their environment. If the fungi are present as free-living organisms (floating or swimming in the stream water, or attached to organic or inorganic matter in the bottom sediments where they may assist in the formation of slimes on the stones, or in the stream bank muds), they may be actively growing spore-producing thalli, or only resting spores. Because of the large numbers recovered in spring, autumn, and winter, they are more likely to be metabolizing organisms than resting spores deposited at the point where the sample was collected by precipitation from the air, or by runoff from nearby soils in which they were active members of the soil population. Once the spores are in the stream, an adequate supply of nutrients is available to them for metabolic activities of relatively constant but low rate.

If it is assumed that even a small amount of organic carbon is readily available to the fungi, and that if additional carbon exists in the form of solids derived from natural pollution such as leaves, bits of organic matter, or even sewage, then there appears to be an adequate supply of carbon available for fungal growth. If some of the carbon is present in the form of proteinaceous material or amino acids as well as carbohydrates, an adequate supply of nitrogen would also be present.

If these conditions form at least/⁸part of the environment of the fungi, then the organisms are in an environment as favorable as the dilute artificial sewage used in the tests of the effect of fungi on BOD (Cooke, et al., 1956). In this work it was found that at low pH levels fungi reduced the BOD of artificial sewage adjusted to a pH of 2.9 rather quickly.

The wide variations in iron content of samples tested and the variations in pH levels indicate that, at least in some of the samples, carbon and iron may be tied up in the form of organically stabilized colloidal iron, which, when precipitated out of the stream water or on the bank, may result in at least some of the yellow boy deposited by these streams. In addition, samples from some of the stations contained water that on settling of all sediments and particulate matter, remained yellowish or yellow rather than becoming clear. Such samples could have come from waters that passed through heavily wooded areas from which the stream picked up quantities of humic acids, which, when complexed with the iron, or some of its forms, yielded a humic acid - colloidal iron complex. This latter factor needs further study in connection with the interpretation of the problem of acid mine drainage.

Wilson, et al. (1964), reported that yeasts isolated from the Monongahela River are capable of breaking down organic matter or sewage solids present in the river water. If the BOD of the river water is lower at some point downstream from a point of sewage discharge because of the activity of fungi of any type rather than a mere dilution or sedimentation factor, and if other organisms usually associated with the

the reduction of BOD in waters are absent, then the fungi that are present are probably active in such removal and are probably similar to the organisms mentioned by Ruchholt, et al. (1940). These organisms may also be involved in the development of floccules of organically stabilized colloidal iron since their surfaces are covered with a thin layer of mucilaginous material. Normally, this is a protective device against desiccation and possibly a physiologically active device that holds food particles or molecules in position during the absorption process.

Molds and yeasts are usually thought of as requiring acid conditions for growth. Prior to the use of rose bengal, a vital stain, and antibiotics, for the reduction in rate of growth of fast-growing fungi, and for the elimination of bacteria from isolation plates, the usual procedure for encouragement of fungal populations from materials of most types was acidification of the medium with lactic or tartaric acid. In the Handbook of Biological Data, Spector (1956) indicates that the general category "molds" has an optimum pH of 4.0-5.8 and a pH range of 1.5-8.5, and "yeasts" has an optimum pH of 3.8-6.0 and a pH range of 2.5-8.0, whereas bacteria have an optimum pH of 6.5-7.5 and a pH range of 3.8-12.0. The use of ranges of these types for the entire group of organisms found in these three categories is most misleading.

Lily and Barnett (1951) indicate that the pH ranges of fungi vary from species to species. The optimum pH may differ for growth and for sporulation stages in the life cycles. The pH of the growth medium may change during the growth of the culture unless the medium is highly

buffered. The net change in pH results from the interaction of the utilization of cations, anions, the formation of acids from metabolites (especially carbohydrates), and the formation of bases (especially ammonia) from amino acids and proteins. Cochrane (1958) lists 34 species of fungi with optimum pH range values. He discusses a number of ways in which various components of a medium may change the availability of a nutrient to a fungus. Geotrichum sp. (probably G. candidum) is listed as having an optimum pH of 3.0.

In his study of Geotrichum candidum, Jones (1964) surveyed the strain with which he worked in relation to growth in a glucose-peptone medium at several pH levels. His experiments were carried out in aerated flasks and lasted for 200 hours. The results of his study are as follows:

<u>Initial pH</u>	<u>Acclimatization lag. hour</u>	<u>Best specific growth rate period</u>	<u>Final pH</u>
3.15	72	100-125	3.05
5.1	15	25-75	2.6
7.35	15	25-50	3.1
9.75	20	25-75	7.6a
11.9	66	75-100	9.5b

a - pH dropped to 5.6 at 125 hours. b - low point of 8.6 was reached at 70 hours.

Using the fungi Aureobasidium pullulans and Phialophora jeanselmei, Cooke and Matsuura (1963) obtained a variety of pH responses. After two weeks growth in agar culture with the agar initially at a pH of 5.7-6.2, depending on the batch, 82 strains of A. pullulans gave a pH range on both glucose and sucrose of 2.7-5.5, and 23 strains of P. jeanselmei on both types of sugar showed a pH range of 3.6-5.5; pH values of intermediate periods were not recorded. A strain of A. pullulans was grown

in media with varying amounts of glucose. The initial pH of all media was 6.1, and the growth continued for 29 days. In the presence of 0.1% glucose there was only a partial removal of glucose, but after 12 days the pH was 3.4 and at the end of the experiment the pH returned to 6.2. In 0.5% glucose there was only partial removal of glucose with 12-day and final pH values of 3.4. In 2.0% glucose there was nearly complete removal of glucose with pH values for the same periods of 4.0 and 3.6. In 4.0% glucose, removal was complete and the pH values were 4.8 and 3.2. In 7.5% glucose, removal was complete and pH values were 5.9 and 3.6. Amounts of growth obtained in the last three sets reached as high as more than a gram of dry weight of tissue. Residual nitrogen was not determined.

In an A. pullulans shaken culture, with an initial pH of 5.5, after 4 days, the 40 strains tested raised the pH of the medium to 6.9-8.9. After 22-25 days, 23 strains of A. pullulans in a Phenol red - destrose fermentation tube experiment with an initial pH of 7.5 yielded no fermentation and a final pH range of 5.6-7.5.

The cited laboratory studies indicate that at least three species of ubiquitous fungi can adjust fairly readily to various culture conditions and pH ranges. In these studies the fungus itself produced or was responsible for the pH change noted in the medium. In natural or artificial assemblages of organisms from which fungi have been isolated, this is not necessarily the case because the fungus is introduced to an environment that it can influence to the extent of removing certain fractions useful to its metabolic processes, but first available to its exoenzyme system. During the process of removal the environment may be modified drastically if the fungus is the only member of the population, but usually the

population is highly complex, and conditions encountered in the laboratory cannot be assumed to apply.

A random sampling of habitats from which samples of soils, waters, sewages, and similar materials have been obtained is presented in Table 31. The type of habitat, number of samples, and pH ranges of these samples are given. The lists of fungi isolated from these samples are quite similar to those isolated from the samples obtained for the acid mine-drainage study. In fact, many of the species listed among the 20 commonest species and species groups in the latter study were among those listed for these samples.

A list of 20 species or species groups of fungi found in all three of the sampled acid mine-drainage areas and in all three of the seasons in which samples were studied, is given in Table 32. The list includes most of the species that were commonly isolated from the samples listed in Table 31. Fewer of these species appeared in samples with higher pH ranges than came from areas with heavy concentrations of salts in the soils and river bottom muds.

That fungi can readily adapt to a wide variety of habitat factors, reducing the element of surprise at finding the same species in a wide variety of river bank, soil, and sewage samples, is attested to by daily contact in the laboratory. Everyone is familiar with the fact that stock solutions of chemicals are readily contaminated by fungus growths. Presumably these stock solutions are prepared, stored, and used with the greatest care for chemical cleanliness. Yet one-tenth normal HCl solutions (pH theoretically 1.0) have yielded Penicillium janthinellum; sodium

Table 31. Habitats Sampled for Fungus Populations

Location	Number of Samples	pH Range
Miscellaneous Colorado and Utah river bottoms	15	6.9-8.8
Soils at bases of plants on a salt desert, Utah	5	8.0-9.4
Conifer woodland soils on granites, Oregon	5	5.5-6.3
Conifer woodland soils on volcanic rocks, Calif.	10	4.5-6.5
Conifer woodland soils on volcanic rocks, Calif.	10	5.5-7.4
Polluted stream, Lafayette, Alabama	5	6.5-7.9
Milwaukee, Wisc., sewage treatment plant	10	5.5-6.2
Milwaukee River, adjacent to above	1	6.9
Westby, Wisc., furrow type sewage treatment and percolation plant	13	6.3-7.5
Hamilton, Ohio, modified activated sludge plant	10	6.9-7.4
Hamilton, Ohio, vacuum filter cake	3	11.7-11.9
Rockford, Illinois, trickling filter plant	5	7.5-7.6

Table 32. Species of Fungi occurring in all Sampled Localities on All Dates - Acid Mine Drainage Study

Aureobasidium pullulans
Candida parapsilosis
Cephalosporium spp.^a
Fusarium oxysporum^b
Geotrichum candidum^b
Gliocladium roseum^b
Mucor spp. (including M. hiemalis)^{a, b}
Penicillium spp.^a
Penicillium janthinellum
Penicillium lilacinum^b
Phialophora jeanselmei
Phoma spp.^a
Phoma herbarum
Rhodotorula spp.^a
Rhodotorula glutinis
Rhodotorula mucilaginosa
Torulopsis candida
Torulopsis famata
Trichoderma viride^{a, b}
Unidentified filamentous fungi^a

^aSpecies groups that include more than one species. In the case of M. hiemalis this may be the predominant member of the group of unidentified cultures. In the case of Trichoderma viride there may be as many as three species in the complex given this species name.

^bSpecies isolated by Dr. Rolf Siepmann from the estuarine muds in the vicinity of Hamburg, Germany. His samples fell within a pH range of 7-8.

propionate (a bread preservative) solutions have yielded Aureobasidium pullulans; and Cephalosporium sp. has been recovered from a 100-ppm standard solution of butyric acid (theoretical pH 3.9). The list could be extended indefinitely if contaminated solutions were retained long enough to catalogue the contaminants.

From the evidence presented above it is not surprising that a number of fungi (Table 33) have become acclimated to the acid mine-drainage habitat and that viable cells can be recovered in considerable numbers from water, stream bottom sediments, and stream bank soils that have received precipitates and sediments from such waters. Because these waters are continually receiving varying quantities of acids from upstream sources as a result of oxidative processes with or without the aid of bacterial action on the mineral contents of opened coal measures, it is most likely that the fungi themselves can only tolerate the environment rather than decrease the acidity to any great extent by their metabolic activities.

Apparently true fungi are not directly involved in transformations leading to the type of pollution referred to as "acid mine-drainage", or to the reduction in acidity of the habitats in which they can survive and grow.

Table 33. Fungi observed in isolations from Acid Mine Drainage streams

Species	Observation Number			
	Total	Hocking	Morgantown	Elkins
Undetermined Filamentous Fungi	1136	362	291	441
<u>Penicillium</u> spp.	1020	348	270	273
<u>Mucor</u> spp.	431	146	132	152
<u>Trichoderma viride</u>	430	212	88	121
<u>Phialophora jeanselmei</u>	243	83	71	84
<u>Cephalosporium</u> spp.	194	65	62	61
<u>Penicillium janthinellum</u>	171	51	72	46
<u>Phoma</u> spp.	137	45	49	39
<u>Geotrichum candidum</u>	116	24	45	46
<u>Penicillium lilacinum</u>	111	33	49	26
Undetermined White Yeasts	103	23	32	42
<u>Aureobasidium pullulans</u>	98	22	27	46
<u>Torulopsis famata</u>	84	13	35	36
<u>Candida parapsilosis</u>	78	19	32	27
<u>Mucor hiemalis</u>	74	34	6	34
<u>Torulopsis candida</u>	74	17	32	25
<u>Gliocladium roseum</u>	69	37	16	16
<u>Aspergillus fumigatus</u>	68	8	31	28
<u>Candida krusei</u>	65	23	32	10
<u>Rhodotorula glutinis</u>	65	25	25	15
<u>Zygorhynchus woelleri</u>	65	22	18	23
<u>Penicillium ochrochloron</u>	63	5	5	53
<u>Fusarium oxysporum</u>	62	17	34	11
<u>Cladosporium cladosporioides</u>	58	24	13	11
<u>Rhodotorula mucilaginosa</u>	56	15	23	18
<u>Candida curvata</u>	49	9	18	22
<u>Penicillium martensii</u>	46	4	12	30
<u>Aspergillus flavus</u>	45	11	26	6
<u>Aspergillus niger</u>	44	11	9	24
<u>Torulopsis</u> sp.	39	5	21	13
<u>Trichosporon cutaneum</u>	39	6	13	20
<u>Alternaria tenuis</u>	37	20	7	7
<u>Penicillium javanicum</u> Ser.	37	10	16	11
<u>Phoma herbarum</u>	37	22	11	3
<u>Cryptococcus laurentii</u>	29	10	7	12
<u>Epicoccum purpurascens</u>	28	10	11	6
<u>Rhodotorula texensis</u>	28	8	4	16
<u>Rhodotorula</u> spp.	27	8	5	5
<u>Fusarium solani</u>	26	15	8	3
<u>Penicillium commune</u>	26	1	14	11
<u>Penicillium nigricans</u>	26	7	14	5
<u>Penicillium chrysogenum</u>	25	13	9	2
<u>Penicillium charlesii</u>	24	21	1	2
<u>Aspergillus</u> spp.	23	4	13	6
<u>Mucor angulisporus</u>	23	6	5	12
<u>Rhizopus</u> spp.	23	12	2	9
<u>Torulopsis glabrata</u>	22	4	3	15
<u>Penicillium thomii</u>	21	8	4	9
<u>Penicillium variabile</u>	20	15	4	1
<u>Cladosporium herbarum</u>	19	3	14	2
<u>Myrothecium verrucaria</u>	19	8	3	7
<u>Penicillium purpureogenum</u>	18	4	4	10
<u>Candida humicola</u>	18	8		10
<u>Penicillium expansum</u>	17	1	5	11
<u>Aspergillus versicolor</u>	16	7	8	1
<u>Gliocladium catenulatum</u>	16	5	3	8

Table 33. P. 2

<u>Paecilomyces varioti</u>	16	3		13
<u>Gliocladium telioscens</u>	15	3	11	1
<u>Torulopsis stellata</u>	15	8	3	4
<u>Fusarium</u> spp.	14	3	8	3
<u>Penicillium funiculosum</u>	14	9	1	4
<u>Phialophora</u> spp.	14	7	4	3
<u>Aspergillus ochraceus</u>	13	8	5	
<u>Candida guilliermondii</u>	13	4	7	2
<u>Gliocladium</u> spp.	13	5	4	4
<u>Penicillium pulvillorum</u>	13		2	11
<u>Saccharomyces cerevisiae</u>	13	4	3	6
<u>Torulopsis aeria</u>	13	1	2	10
<u>Torulopsis datilla</u>	13	3	6	4
<u>Aspergillus ustus</u>	12	5	4	3
<u>Candida</u> spp.	12	1	8	3
<u>Penicillium simplicissimum</u>	12	9	3	
<u>Pichia guilliermondii</u>	12	8	2	2
<u>Achlya dubia</u>	11	6	4	1
<u>Fusarium aqueductum</u>	11	8	1	1
<u>Penicillium vermiculatum</u>	11		6	5
<u>Trichoderma alba</u>	11	1	2	8
<u>Achlya klebsiana</u>	10	5	3	2
<u>Fusarium roseum</u>	10	5	2	3
<u>Gliocladium fimbriatum</u>	10	5	5	
<u>Leptosporium</u> spp. ss. lat.	10	5	2	3
<u>Trichosporon</u> spp.	10	1	2	2
<u>Trichosporon pullulans</u>	10	1	7	2
<u>Verticillium</u> spp.	10	6		4
<u>Achlya americana</u>	9	9		
<u>Candida intermedia</u>	9	1	6	2
<u>Candida gucodermis</u>	9	2	1	6
<u>Penicillium solitum</u>	9	1	1	7
<u>Achlya</u> sp.	9	4	4	1
<u>Gliomastix convoluta</u>	8	4		3
<u>Penicillium corymbiferum</u>	8	1	4	3
<u>Penicillium frequentens</u>	8	1	6	1
<u>Penicillium implicatum</u>	8	1	2	5
<u>Verticillium lateritium</u>	8	2	1	5
<u>Saprolegnia</u> spp.	8	1	4	3
<u>Aspergillus sydowii</u>	7	6		1
<u>Candida tropicalis</u>	7	4	1	2
<u>Chrysosporium pannorum</u>	7	5	2	
<u>Cladosporium</u> sp.	7	6	1	
<u>Mucor plumbeus</u>	7		4	3
<u>Candida solani</u>	6		1	5
<u>Candida utilis</u>	6		2	4
<u>Curvularia geniculata</u>	6	5	1	
<u>Gongronella butleri</u>	6	1		5
<u>Paecilomyces</u> spp.	6		1	5
<u>Penicillium kanusacinskii</u>	6	3	1	2
<u>Penicillium rubrum</u>	6	1	3	2
<u>Penicillium sclerotiorum</u>	6	4	2	
<u>Pestalotia heterocornis</u>	6	1	3	2
<u>Cladosporium sphaerospermum</u>	5	3		2
<u>Cryptococcus albidus</u>	5	3	2	
<u>Curvularia lunata</u>	5	3	2	
<u>Oidiodendron</u> sp.	5		1	
<u>Paecilomyces marquandii</u>	5	3	2	
<u>Penicillium crustosum</u>	5	3		2
<u>Trichosporon capitatum</u>	5	1		4

Table 33. P. 3

<u>Aspergillus chevalieri</u>	4	2	1	1
<u>Colletotrichum gloeosporioides</u>	4	3		1
<u>Coniothyrium spp.</u>	4		2	
<u>Penicillium canescens</u>	4		2	2
<u>Penicillium steckii</u>	4	1	3	
<u>Phialocephala spp.</u>	4	2	1	1
<u>Pythium spp.</u>	4	4		
<u>Torulopsis inconspicua</u>	4		1	3
<u>Penicillium wakemanii</u>	4		4	
<u>Absidia glauca</u>	3	2	1	
<u>Achlya diffusa</u>	3	2	1	
<u>Aureobasidium mansonii</u>	3		2	1
<u>Candida scottii</u>	3		1	2
<u>Chloridium chlamydosporum</u>	3			2
<u>Cryptococcus diffluens</u>	3	2	1	
<u>Rhizoglyphus grisea</u>	3	1	2	
<u>Penicillium brefeldianum</u>	3	2	1	
<u>Penicillium decumbens</u>	3	3		
<u>Penicillium herquesi</u>	3	2	1	
<u>Rhizopus chinensis Ser.</u>	3			1
<u>Torulopsis colliculosa</u>	3		1	2
<u>Penicillium diversum</u>	3			3
<u>Sepedonium sp.</u>	3		1	2
<u>Achlya glomerata</u>	2			2
<u>Aspergillus restrictus</u>	2	1	1	
<u>Candida rugosa</u>	2	2		
<u>Chaetomella sp.</u>	2		2	
<u>Coniothyrium fuckelii</u>	2			2
<u>Cryptococcus luteolus</u>	2			
<u>Dictyuchus sp.</u>	2		2	
<u>Doratomyces stemonitis</u>	2	2		
<u>Isaria farinosa</u>	2	1	1	
<u>Monilia sp.</u>	2	2		
<u>Mucor alternans</u>	2	1		1
<u>Mucor racemosus</u>	2		1	1
<u>Papularia sp.</u>	2			2
<u>Penicillium puberulum</u>	2	2		
<u>Phaeoisaria sp.</u>	2			2
<u>Phialophora lagerbergii</u>	2			
<u>Saprolegnia littoralis</u>	2		1	1
<u>Scopulariopsis parvula</u>	2		1	1
<u>Sporotrichum epigaeum var. terrestre</u>	2	2		
<u>Torula sp.</u>	2		2	
<u>Torulopsis versatilis</u>	2	1		1
<u>Aphanomyces sp.</u>	2	2		
<u>Helminthosporium sp.</u>	2		1	1
<u>Absidia cylindrospora</u>	1	1		
<u>Actinomyces elegans</u>	1		1	
<u>Arthrinium arundinis</u>	1	1		
<u>Aspergillus clavatus</u>	1	1		
<u>Aspergillus fischeri</u>	1	1		
<u>Aspergillus flavipes</u>	1	1		
<u>Aspergillus tamarii</u>	1	1		
<u>Aspergillus terreus</u>	1	1		
<u>Basidiomycete</u>	1			
<u>Candida pseudotropicalis</u>	1	1		
<u>Chaetomium globosum</u>	1		1	
<u>Cladosporium avellaneum</u>	1	1		
<u>Cladosporium elatum</u>	1	1		

Table 33. P. 4

<u>Cladosporium macrosporum</u>	1			1
<u>Cunninghamella elegans</u>	1	1		
<u>Curvularia sp.</u>	1			
<u>Cytospora sp.</u>	1			
<u>Fusarium acuminatum</u>	1	1		
<u>Fusarium moniliforme</u>	1		1	
<u>Fusarium oxysporum var. redolens</u>	1		1	
<u>Fusarium sambucinum</u>	1	1		
<u>Hansenula suaveolens</u>	1	1		
<u>Kloeckera apiculata</u>	1	1		
<u>Memmoniella echinata</u>	1			
<u>Monosporium apiospermum</u>	1	1		1
<u>Mortierella isabellina</u>	1	1		
<u>Nectria flava-virens stat. conid.</u>	1		1	
<u>Paecilomyces elegans</u>	1		1	
<u>Penicillium aculeatum</u>	1			1
<u>Penicillium cyclopium</u>	1	1		
<u>Penicillium islandicum</u>	1		1	
<u>Penicillium oxalicum</u>	1	1		
<u>Penicillium piscarium</u>	1		1	
<u>Penicillium velutinum</u>	1	1		
<u>Periconia sp.</u>	1		1	
<u>Pestalotia versicolores Group</u>	1	1		
<u>Phialocephala dimorphospora</u>	1			1
<u>Preussia microspora</u>	1	1		
<u>Prototheca moriformis</u>	1			
<u>Rhizopus arrhizus</u>	1	1		
<u>Saccharomyces sp.</u>	1	1		
<u>Saccharomyces rosei</u>	1	1		
<u>Scopulariopsis asperula</u>	1		1	
<u>Scopulariopsis brevicaulis</u>	1	1		
<u>Scopulariopsis brumptii</u>	1	1		
<u>Selenophoma sp.</u>	1	1		
<u>Septoria sp.</u>	1			
<u>Sphaeronema spinella</u>	1			
<u>Sporotrichum sp.</u>	1			1
<u>Stemphylium sp.</u>	1			1
<u>Stemphylium botryosum</u>	1			1
<u>Stilbellaceae sp.</u>	1			1
<u>Syncephalastrum racemosum</u>	1			
<u>Thraustotheca clavata</u>	1		1	
<u>Trichosporon behrendii</u>	1			1

VI. Fungi in Sewage Treatment Systems

A. Introduction

In the introductory literature survey it was pointed out that in the earlier phases of the study of the microbiology of sewage treatment systems, the fungi were considered to be nuisance organisms. In trickling filters they were known to cause ponding; in the activated sludge tanks they were known to cause bulking. The basic reasons for their causing ponding or bulking were unknown and ignored.

To determine whether fungi occur in sewage and polluted water, and whether they are potentially of importance in the treatment of sewage, it became necessary to initiate a survey of a sewage treatment plant in order to determine those parts of the plant or those stages of the treatment process in which fungi could be expected to be found. If fungi could be found in sufficient quantity in any part of the treatment process it might develop that they were contributing materially to the biological treatment of sewage at that particular stage in the process.

To determine techniques which could be useful and relatively easily manipulated in the laboratory, scrapings of surface slimes from the stones of the Glendale, Hamilton County, Ohio, trickling filter were studied. Several techniques were tried, and as indicated above, that of the soil scientist was adapted. It was thought that a large plant with several readily sampled stages in the treatment process would yield information of the type desired, and the Dayton, Montgomery County, Ohio, sewage treatment plant was chosen as a sampling area. Without the cooperation of the superintendent, L. T. Hagerty, and his successors, and his assistant, R. H. Calihan, and various

members of the personnel of the plant, the sampling could not have been as complete nor as readily and speedily carried out during each of the 19 visits.

A study by Becker and Shaw (1955) was developed on the basis of materials obtained from the Pullman, Washington, and Moscow, Idaho, sewage treatment plants. Both plants are of the secondary type utilizing trickling filters. Conclusions were drawn largely on the basis of findings in effluents from the several processes.

Elsewhere, interest in the role of fungi in the sewage treatment plant has been limited to their activity as nuisance organisms or as members of certain trickling filter slimes, especially in England.

B. The Dayton, Ohio Sewage Treatment Plant *

The Dayton, Ohio sewage treatment plant is located south of the City of Dayton on the west shore of the Great Miami River. At the start of sampling in the spring of 1952 and throughout the following year during 12 monthly samplings, gravity flow sewage from the city was pumped to a grit removal chamber from which it was piped directly to primary settling in a series of Imhoff tanks. The supernatant from the Imhoff tanks was fed directly to the trickling filters while the sludge was piped to a series of unheated and heated digesters for secondary digestion. In 1952 and 1953 the low rate trickling filters received a maximum loading continuously, while the high rate trickling filters were reserved for the excess over that which the low rate filters could handle. The high rate filters operated continuously, but they approached maximum capacity only during peak loading of the plant.

*Reproduced with permission from Proceedings of the 13th Industrial Waste Conference, Purdue University Engineering Bulletin 43:26-45. 1958. (1959).

Following digestion, sludges were drawn from digesters to sand-based drying beds, some under glass, some in open beds. Sludges were sampled in place on the beds in the freshly poured state, in a medium dry state (2 weeks to 2 months after being poured), and in the completely air-dried state just before they were removed from the beds for further treatment. Following air-drying the sludge was broken up, passed by conveyor belt through a heated chamber at approximately 300°F, pulverized, stored, sacked, and sold as "Or-So-Con", an organic soil conditioner. In addition to 9 sets of this material, sludges being dried in vacuum filters, and sludges on the drying beds including rain-soaked and high calcium-content sludges were sampled. These sludges as well as Imhoff tank scums and detritus, which were also sampled, are discarded by incineration.

Sampling stations in the sewage treatment process varied as the processes were altered. At first, sampling included raw sewage after detritus had been removed, settled sewage as it entered the trickling filters, oxidized sewage as it was discharged from both the high and low rate trickling filters, sewage as it was found in the secondary settling basin, and final effluent in the channels through which it was discharged to the Great Miami River.

Samples were also taken from the bottom of the Imhoff tanks after sludge had been held for approximately 7 days, from the bottom of the 30-foot deep secondary sludge digesters where it had been held for approximately 30 days at 95°F, and after it had been poured on the drying beds.

After the addition of a preaeration system, detritus from the new grit removal chambers, raw sewage entering the preaeration tanks, sewage near the effluent from the preaeration tanks, and primary settled sewage

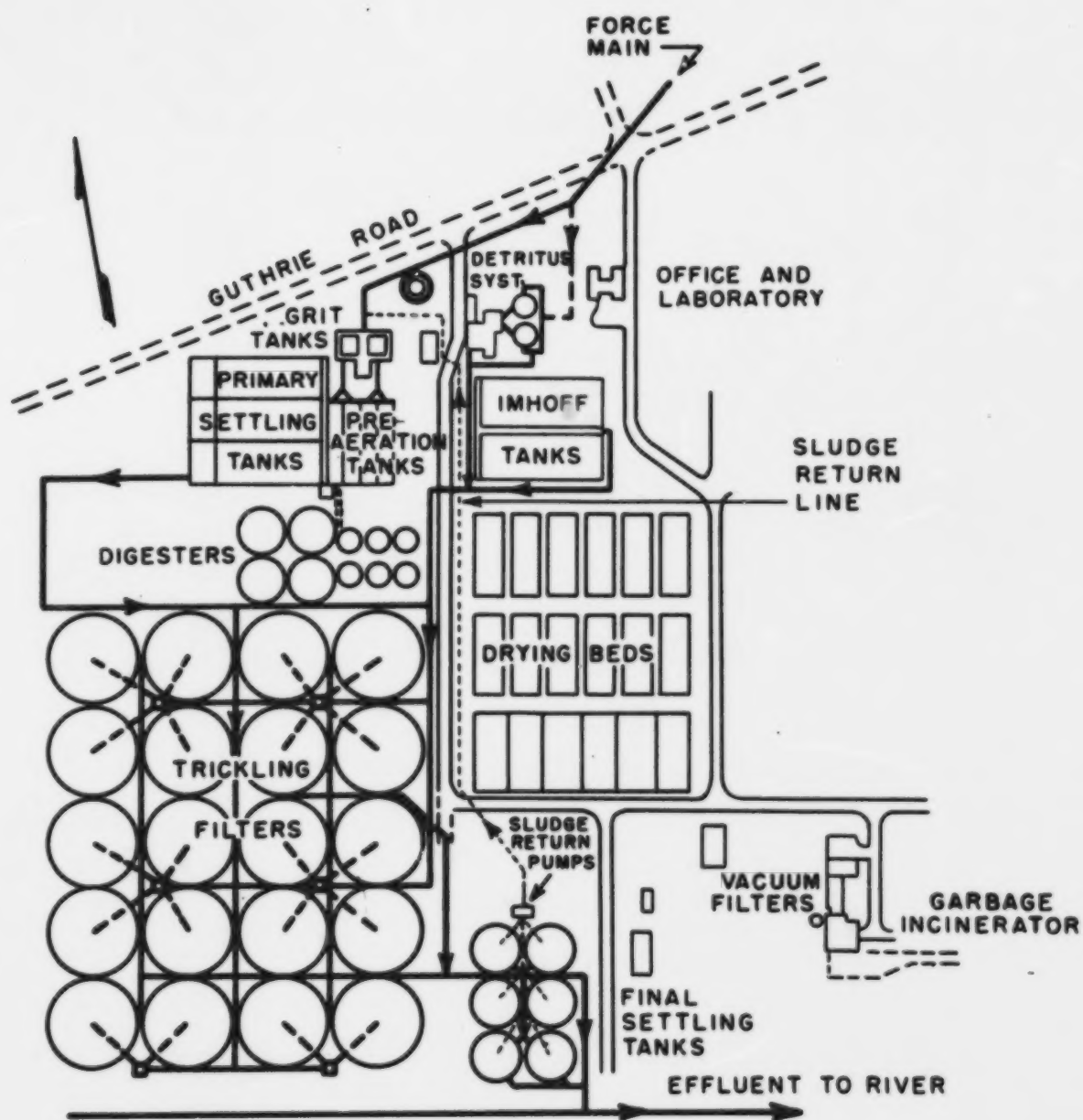
and primary settled sludge were sampled. A flow chart is presented of this system (Fig. 7).

Grab samples obtained in glass bottles, metal cans or plastic vials were processed on return to Cincinnati following techniques described above (Chapter II).

Results

Results of plating samples obtained from 27 types of habitat on 19 sampling dates are given in Tables 34-37. These are divided into four types of materials representing (1) the liquid system based on the liquids as they passed through the system; (2) the solids system based on the sludges as they passed through several treatment phases; (3) the permanent populations based on the slimes on the stones of the surface of the filters; and (4) the discarded materials including detritus, scums and vacuum filter cake, all of which are incinerated.

Results are presented as percentages of total numbers of colonies of fungi isolated from any one of the 27 types of habitat. At the head of each column is indicated the number of samples taken and the number of times each habitat was available for sampling. If a process was put into use at the end of the 18th sampling period, it could have been sampled only once; or if a process was available for sampling only once because of novelty or because of mechanical difficulty, it was sampled only once. The total number of colonies obtained from a particular habitat, on which the percentage value is based, is presented. To arrive at a more realistic value this total should be multiplied by the dilution factor to obtain the number of colonies present in 1 ml of original sample. If one wished to correct for the number of colonies present in a gram dry weight of original sample,



SCALE = 1/4" = 50' (APPROX.)

FLOW CHART - DAYTON, OHIO, SEWAGE TREATMENT PLANT

FIGURE 7

Table 3⁴. Dayton, Ohio, sewage treatment plant.

Additional species

Table 35. Dayton, Ohio, Sewage treatment plant. Percent occurrence of fungus species -

State of Solids Location Sampled	Solids system									
	Digestion					Drying				
	Inhoff Tank Sludge	Primary Settled Sludge	Primary Digester Sludge	Secondary Digester Sludge	Composite Digester Supernatant Sludge	Freshly Poured Sludge	Medium d., Sludge	Medium dry, high Ca Sludge	Air Dry Sludge	Heat Dry Sludge re-wet by Rain
Times sampled/times visited	12/14	5/5	1/5	17/19	1/1	16/19	17/19	1/1	18/19	9/19
Dilution factor	1:1000	1:1000	1:1000	1:1000	1:1000	1:10000	1:10000	1:10000	1:10000	1:10000
Total colonies counted	965	1001	93	1158	110	1522	4213	19	17,759	844
Fungus Species	20.3	23.9	55.9	23.9	54.6	15.4	43.6	10.5	51	844
White yeasts										
<i>Clostridium candidum</i>	19.6	32.9	6.4	14.3	10.0	6.0	2.4	68.4	1.3	58.8
<i>Penicillium soureductum</i>	4.4	1.0	3.2	3.2	3.7	6.9	2.5	5.3	.5	5.9
<i>Phialophora lecanioides</i>	8.8	8.0	7.5	22.0	12.8	29.0	15.4		.9	2.0
<i>Penicillium ochrochrocon</i>	2.5	1.6	2.0	2.0		5.7	1.5		.2	1.5
<i>Penicillium lilacinum</i>	7.5	2.9	2.1	6.7	1.9	10.2	2.6		.6	11.6
<i>Rhodotorula</i> spp.	2.3	3.1	1.1	2.5	2.8	5.7	5.1		18.4	2.8
<i>Aspergillus versicolor</i>	1.3	1.7		.9		.7	.2		.05	.6
<i>Trichosporon</i> sp.	.1						24.2		60.9	38.0
<i>Aureobasidium pullulans</i>	7.0	5.8		.9		3.0	1.2		.3	2.0
<i>Phoma</i> spp.	1.0	.4		4.4	.9		.1	5.3	.9	2.0
<i>Trichoderma viride</i>	2.2	1.4		2.0		1.4	.3		.05	2.0
<i>Cladosporium cladosporioides</i>	1.5	11.0		.9		1.1	.2		.3	.8
<i>Moniliaceae</i> spp.	.7	2.2	1.1	1.1	1.9	.3	.3	10.5	1.7	1.1
<i>Penicillium</i> spp.	2.8	1.7	3.2	1.5	3.7	2.2	.4		.2	5.9
<i>Aspergillus fumigatus</i>	.8		1.1	1.0	1.9	.7	.2		2.8	5.3
<i>Aspergillus ochraceus</i>	.6			.2		.2	.07		5.4	20.5
<i>Penicillium funiculosum</i>	1.5			2.0		1.3	.4		.03	.2
<i>Aspergillus clavus</i>	.3			.8		.2	.07			1.3
<i>Aspergillus versutus</i>	.4					.1	.07		.006	.1
<i>Mucor</i> spp.	1.4	.1	5.4		.9	.5	.1		.06	7.6
<i>Mucor fragilis</i>	1.4	.3		.6		.7	.2		.2	1.1
<i>Aspergillus niger</i>	1.3	.2		.7		.7	.2		.05	7.5
<i>Aspergillus fischeri</i>										.6
<i>Mucor plumbeus</i>	.1		1.1	.2	.9	.4	.09		.08	
<i>Fusarium</i> spp.	3.3			.2		.07	.05		.01	
<i>Penicillium varioti</i>	.6		1.1	.3		.5	.02		.01	.1

Table 35. p. 2

Cephalosporium spp., Subbaromyces splendens, Dematiaceae spp., Alternaria tenuis, Verticillium lateritium, Allescheria bordii, Aspergillus sydowii, A. flavipes, A. candidus, A. terreus, A. unguis, A. wentii, Coniothyrium fuckelii, Penicillium chrysogenum, P. oxalicum, P. lanthinellum, P. stictit, P. cyclopium, P. puberulum, P. martensii, P. piscarium, P. roquefortii, P. palitans, P. citrinum, P. daleae, P. herquei, P. corylophilum, P. velutinum, P. raistrickii, P. purpureogenum, P. commune, P. cyanocephalum, P. rolfsii, P. pulvillorum, P. spinulosum, P. variabile, P. expansum, Fusarium oxysporum, F. solani, F. roseum, Gliomastix convoluta, Absidia ramosa, Mucor racemosus, M. nr. saturninus, Doratomyces stenonitis, Gliocladium roseum, Stachybotrys atra, Rhizopus arrhizus, R. oryzae, R. rhizopodiformis, R. spp., Ascomycetes, Acremonium sp., Syncephalastrum racemosum, Zygoryhynchus weilleri, Memnoniella echinata, Endomyces sp., Helminthosporium sp., Haplographium sp., Pseudoplea americana, Styragus sp., and Hemicola grisea.

Table 36. Dayton, Ohio, Sewage treatment plant, percentage occurrence of fungus species - permanent populations

[illegible]

Table 37. Dayton, Ohio, sewage treatment plant. Percent occurrence of fungus species - Discard Materials

Table 37. Dayton, Ohio, sewage treatment plant.					
Water Sampled	Detritus	Tahoff Tank	Scum Primary Settling Basin	Cake	Vacuum Filter Effluent
Times Sampled/Times Visited	3/19	10/14	1/5	1/1	1/1
Dilution Factor	1:1000	1:1000	1:1000	1:10000	1:100
Total colonies counted	359	1075	123	28	16
Fungus species					
White yeasts	24.5	21.7	48.8	3.6	6.2
<i>Clostridium sandium</i>	33.7	16.9	5.7		12.5
<i>Fusarium aqueductum</i>	3.0	3.2	1.8		
<i>Phialophora lecaniella</i>	6.7	9.1	9.8	7.1	56.2
<i>Penicillium ochrochloron</i>	1.9	2.8		67.9	
<i>Penicillium lilacinum</i>	2.5	5.5	4.9	3.6	6.2
<i>Penicillium</i> spp.	1.1	3.1	11.4		12.5
<i>Aspergillus</i> spp.	.03	1.2	.8	10.7	
<i>Aspergillus versicolor</i>	14.5	6.4		10.7	
<i>Aspergillus pullulans</i>	.8	.7			
<i>Trichoderma viride</i>	.3	2.1			
<i>Gladosporium cladosporioides</i>	3.0	1.5	3.3		
<i>Penicillium</i> spp.	1.7	1.7	4.9		
<i>Penicillium</i> spp.			3.3		
<i>Aspergillus fumigatus</i>	.8	1.2			
<i>Penicillium funiculosus</i>	.6	1.6	1.6		
<i>Aspergillus flavus</i>		1.2	1.6		
<i>Aspergillus utahus</i>		.2	.8		
<i>Aspergillus</i> spp.		1.8			
<i>Mucor fragilis</i>		2.3	.8		
<i>Aspergillus niger</i>		1.1			
Additional species					
<i>Aspergillus ochraceus</i> , <i>A. sydowii</i> , <i>A. flavipes</i> , <i>A. candidus</i> , <i>A. terreus</i> , <i>A. clavatus</i> , <i>A. tamaris</i> , <i>A. natus</i> var. <i>laevis</i> , <i>Mucor plumbeus</i> , <i>Penicillium</i> spp., <i>Subbotomyces splendens</i> , <i>Dematiaceae</i> spp., <i>Alternaria tenuis</i> , <i>Allescheria boydii</i> , <i>Acremonium</i> sp., <i>Verticillium</i> , <i>Lateridium</i> , <i>Fascicomyces varioti</i> , <i>Penicillium chrysogenum</i> , <i>P. variable</i> , <i>P. oxalicum</i> , <i>P. lanthinellum</i> , <i>P. cyclopium</i> , <i>P. puberulum</i> , <i>P. maritimi</i> , <i>P. velutinum</i> , <i>P. purpurescens</i> , <i>Fusarium oxysporum</i> , <i>F. solani</i> , <i>Gliomastix convoluta</i> , <i>Gliocladium roseum</i> , <i>Stachybotrys atra</i> , <i>Botrytis cinerea</i> , <i>Chaetomium</i> sp., <i>Phialophora</i> sp., <i>Microthecium verrucaria</i> , <i>Stilbella</i> sp., <i>Botrytis</i> spp., <i>Chaetomium</i> sp., <i>Phialophora</i> sp., <i>Scedonum thielavioides</i> .					

an additional factor could be used. In liquid samples where less than 0.3 percent of the sample is solid matter, or even in the case of digested sludges where only 4 or 5 percent of the sample is solid material, this latter expression based on oven dry weight is not a realistic approach.

Of the 112 species or categories of fungi listed in this study, 29 are present often enough or in large enough quantity so that they can be considered permanent members of the population. Some of these are unimportant in one or more of the processes in which they were found, and of the remaining species a few may be of considerable importance in the habitats in which they were found. Except for the "white yeasts" and Rhodotorula, the red yeast, none of the categories containing as yet unidentified species can be said to include species of importance to the treatment system.

To supplement information on the permanent populations of the trickling filters, the percentage occurrence of the colonies of fungi on glass slides exposed on the surface of the two filters under consideration is given. These data will also supplement those presented below

(Section C). They help to compare and contrast these two methods of collecting data relative to the populations to be found on the surface stone of trickling filters.

The order in which the species are presented in the tables is the order of their importance in the sewage treatment plant. This was obtained by finding the total number of colonies of fungi isolated from any one sampling station on all sampling dates, taking the percent occurrence of each species or category found in that sampling location, and adding together

the percent occurrence values for each species. On the 19 sampling dates, 27 stations were sampled, but only 3 stations were sampled on all 19 dates. If any one species had been found at all stations on any one date, its percent occurrence would have been scored at 2700. On this basis, the score for the occurrence of the commonest category, unidentified white yeasts, was 650.3. Occurrence scores for the 3 commonest species, represented in 25, 24, and 24 of the sampling stations respectively, was Geotrichum candidum 367.2, Fusarium aquaeductum 257.4, and Phialophora jeanselmei 239.4. Of the 112 species of fungi found in the plant, seven categories had a total occurrence score of more than 100, 22 categories had a total occurrence score of more than 10, and 50 categories had a total occurrence score of more than 1. A species of Trichosporon, a filamentous yeast, was found in only 4 sampling stations yet its occurrence score was 87.2, while Cladosporium cladosporioides, a relatively common mold, was found in 22 sample stations and had an occurrence score of only 60. Thirty-six fungi were found in only one sampling station each. Their occurrence scores ranged from as high as 7.5 to 0.006.

Those species with occurrence scores of less than 1 may all be considered as lymanxes (organisms tolerating aswage habitat) species which have not become established in any phases of the treatment process but are passing through the treatment plant with the flow of sewage and have accidentally been trapped in the sampling process. Such species may have been carried into the plant in the runoff which enters domestic sewage channels, in washing of soiled hands, clothing, floors, walls, fruits, vegetables, or other commodities, or in other ways. They apparently are able to tolerate the conditions under which they have been found even to

the extent of reproducing to a limited extent in some locations such as on trickling filters, the walls of some tanks, or basins, sludge as it is drying, or in other places. Some of those 28 species whose occurrence score lies between 1 and 10, and which have been found in more than 10 sampling stations (13 species) (rarely in fewer), may also be considered lymnaeans, but probably many of them are lymnaeophiles (organisms living in a sewage habitat).

Only two of the 112 species of fungi found were identified as lymbionts, organisms not known to occur in other than sewage treatment plants or in association with other types of excrement. Of these, Ascodemis sphaerospora has been found only once, while Subbaromyces splendens has been found in 8 sample stations with an occurrence score of 5.64. The latter species is very fastidious in its nutrient requirements (Hesseltine, 1953), and its appearance is more accidental than planned, for the isolation medium used probably does not contain all the nutrients required for its optimum growth, but these are as yet unknown. Besides being found in the two types of trickling filters, it has been found in raw sewage, Imhoff tank sludge, sludge digester supernatant, effluent from high rate trickling filters, and the effluent from the sewage treatment plant. With the use of a medium which would be complete for this fungus, its range in sewage treatment processes and in nature could become better known.

On Spore Production by Fungi in Aquatic Habitats

In general, there are among the fungi two principal types of asexual spore production. The true water molds including those Phycomycetes such

as the chytrids, and most of the Oomycetes, reproduce by means of swimming, motile, zoospores. These fungi live in the water, where they are associated with decaying organic debris, such as leaves or sticks, or with living organisms where they may be active parasites of algae, protozoans, or fish. They may be found in soils only when the relative humidity of the air spaces in the soil is at or near 100%. Aquatic Hyphomycetes are known to attack dead organic matter, especially leaves and herbage, in wet places such as roadside ditches, pools and marshes. These fungi are able to develop an elaborate vegetative mycelium under the water but send up to the air specialized hyphae at the tips of which highly specialized spores are produced. These fungi are considered aquatic as the soil is waterlogged during their growth. Other mold-like fungi are able to develop in wet places during the vegetative portion of their life cycles, such as those species which prey on nematodes, amoebae, and other microscopic animals in the litter of the garden or the woodland.

In the laboratory, and in the factory where fungi are put to work in fermentation and other processes for the production of antibiotics, organic acids, or other chemicals, or where fungi are being studied for their physiological properties of many types, dry spores may be added to liquid solutions, the spores will germinate, produce a mycelium, and during the course of the laboratory experiment or commercial run, continue growth in the form of mycelium. In general such types of growth do not sporulate in submerged culture, or sporulation may be very different from that encountered in "dry" culture on a petri dish, or in nature. Usually such mycelial growths occur in the form of balls or masses of mycelium.

The trickling filter may be likened to such a habitat, and the activated sludge tank is very similar, although here the fungus can become attached to any of the large numbers of particles in the sewage rather than developing its mycelial ball independently of particulate matter. On the trickling filter the fungus spore or mycelial fragment can germinate on a stone to which it can be held fast while developing a mycelium which is bathed in a continuous flow of nutrients perhaps relatively dilute. The liquid settled sewage has never been completely analyzed for its content of materials which may be nutrients for the growth which occurs on the filter stone, so this growth may be considered, in part at least, a group of assay organisms for the sewage. For instance, it is known through laboratory experiment that at least one strain of Fusarium aquaeductum requires thiamine for optimum growth, and a strain of Aureobasidium pullulans requires biotin. The tested strains of these two fungi cannot manufacture their own requirements of these two vitamins, yet the former represented nearly one-third of the fungi found on the filter, whether the sampling was the simple scraping of the stone or the placing of sterile surfaces onto which the slime organisms could grow. That the latter species makes up only 3 percent of the fungus growth in either type of sampling is no less significant. The abundance in which both organisms is found on the filters indicates that the supply of thiamine and biotin in the settled sewage is adequate to maintain these fungi at the level required for their growth within the limits of competition with other organisms.

The fact that fungi, in liquid culture in the laboratory and in industry, generally do not produce spores in abundance or typically, is reflected by the reproductive response of many types of fungi found on the trickling filters. This was emphasized by Becker and Shaw (1955) who found fewer fungi in effluents from various types of processes in the sewage treatment plants at Pullman, Washington, and Moscow, Idaho, than in influents. However, growth on the trickling filters at Dayton includes the mycelia of more than 90 species of fungi according to two different types of sampling techniques and 20 of these are found in considerable quantity using either technique.

Of the commoner species on the filters, the type of sporulation is of interest since it may permit the fungus to be carried beyond the filters without the benefit of sloughing, which must be the type of dissemination through which other fungi are spread throughout the plant. It is thought by specialists working with the genus Fusarium that the pigments found in F. aquaeductum lie wholly within the walls of the spores. This species produces the orange to burnt umber coloration so commonly observed on trickling filters throughout the country. If the pigmentation were due solely to coloring matter in the walls of the spores, this fungus would have to produce large numbers of spores in order to give as striking a color to the filters as it does. It has been noted in this laboratory that the mycelium, in clear water mounts, has the same coloration as that of the spore walls. If this holds true for the mycelium in place on the filters, it would account for most of the coloring caused by this fungus. There is no doubt that the fungus produces spores on the

surface of the filter and that through this means it can be carried in the plant effluent and throughout the plant when sludge is returned from the final settling tanks to the plant influent lines.

Geotrichum candidum is a white mold which is abundant in the filter slime. It produces arthrospores by a special type of wall formation which permits the cells of the mycelium to break up upon drying out or upon disturbance. Its white color would be more evident on the filters throughout the year if it were not masked by the green color of the large numbers of algal cells under, among, or on which it grows. Another mold which produces a white color on the filters at some times of the year is Leptomitum lacteus which has been observed on filters but never isolated into pure culture directly from them. Phialophora Heanselmei produces conidia in succession in slime balls from annellophores. A minute hyphal tip protrudes from the spore and from this another spore may be produced. This appears to be a type of budding process. These spores, in liquid culture, appear to reproduce more commonly by yeast-like budding rather than by mycelial formation. It is possible that some of the spores of this species on the filter reproduce by this method.

Aureobasidium pullulans produces a colorless (hyaline) mycelium which may become colored and from the cells of which many radulaspores may be produced from minute spicules at irregular intervals in a manner resembling the production of yeast cells. This is called the "black yeast" but its color is masked by the many other organisms which may be present. Under the conditions of the trickling filter, the species of

Penicillium which have become adapted to life there, especially P. lilacinum and P. ochrochloron, apparently are able to sporulate to a slight extent. Instead of producing typical spore-producing hyphal branches (phialides), these fungi produce small, single, scattered phialides along the hyphae. Spores are produced singly or on short chains on such structures on the filters. The white yeasts, and the red yeast Rhodotorula, reproduce by budding and large colonies of cell's may be produced. These fungi produce little that can be considered as true mycelium. Their nearest approximation is a "false" mycelium made up of loose series of few hypha-like cells. These cells can bud and produce yeast cells under relatively unfavorable conditions. Most of the other fungi present in slime on trickling filters produce spores rarely, if at all, and their methods of reproduction are vegetative. The cells of the vegetative mycelium may be carried from place to place in the washings from filter stones following weak to severe sloughing induced by a temporary lack of water, or by excessive flushing resulting from heavy precipitation.

Discussion

Within the various processes and their effluents in the Dayton, Ohio sewage treatment plant, a relatively large number of species of fungi has been found (Table 38). Most of these fungi are normal inhabitants of the population of soil fungi which are thought to occur in the area. Such fungi may have entered the treatment plant through domestic sewage or industrial wastes. Once inside the plant these fungi found a congenial location in which to live and reproduce or they passed on through the plant, being accidentally picked up as they were being carried by

Table 38. Species of Fungi Observed in the Dayton Sewage Treatment Plant

Species	Observation Number	Sample Dates (21)	Sample Areas (23)
White Yeasts spp.	521	19	23
<u>Geotrichum candidum</u>	403	21	21
<u>Rhodotorula</u> spp.	259	19	21
<u>Penicillium lilacinum</u>	237	21	21
<u>Fusarium aquaeductuum</u>	200	20	22
<u>Phialophora jeanselmei</u>	199	20	21
<u>Penicillium</u> spp.	196	20	15
<u>Moniliaceae</u>	194	16	21
<u>Aureobasidium pullulans</u>	184	16	21
<u>Penicillium ochrochloron</u>	171	16	19
<u>Cladosporium cladosporioides</u>	137	15	18
<u>Mucor fragilis</u>	130	19	17
<u>Trichoderma viride</u>	125	19	17
<u>Aspergillus versicolor</u>	102	14	19
<u>Aspergillus fumigatus</u>	89	16	20
<u>Phoma</u> spp.	87	19	21
<u>Penicillium funiculosum</u>	84	16	17
<u>Aspergillus niger</u>	80	20	15
<u>Candida parapsilosis</u>	78	6	16
<u>Rhodotorula mucilaginosa</u>	74	12	12
<u>Torulopsis fawcettii</u>	70	5	10
<u>Aspergillus flavus</u>	56	15	18
<u>Alternaria tenuis</u>	55	16	15
<u>Mucor plumbeus</u>	55	17	17
<u>Candida krusei</u>	47	3	17
<u>Monosporium apiospermum</u>	38	12	14
<u>Dematiaceae</u>	36	8	10
<u>Aspergillus ochraceus</u>	33	13	14
<u>Paecilomyces variotii</u>	31	14	12
<u>Penicillium oxalicum</u>	30	9	10
<u>Fusarium solani</u>	29	8	9
<u>Penicillium chrysogenum</u>	29	10	11
<u>Verticillium lateritium</u>	29	8	13
<u>Candida guilliermondii</u>	28	2	7
<u>Mucor</u> spp.	27	8	12
<u>Trichosporon</u> spp.	27	6	12
<u>Trichosporon cutaneum</u>	23	8	12
<u>Fusarium oxysporum</u>	22	8	14
<u>Torulopsis candida</u>	22	4	7
<u>Aspergillus natus</u>	21	6	12
<u>Torulopsis</u> spp.	20	1	6
<u>Saccharomyces cerevisiae</u>	19	1	4
<u>Gliomastix convoluta</u>	18	8	10
<u>Subbaromyces splendens</u>	18	7	10
<u>Aspergillus sydowii</u>	17	8	14
<u>Rhodotorula glutinis</u>	16	10	10
<u>Aspergillus flavus</u>	15	7	10
<u>Candida rugosa</u>	15	3	13
<u>Penicillium janthinellum</u>	14	7	8
<u>Torulopsis tsakii</u>	14	1	6
<u>Doratomyces stemonitis</u>	13	4	7
<u>Penicillium wartschii</u>	13	6	7
<u>Gliocladium roseum</u>	12	9	9
<u>Trichosporon pullulans</u>	12	3	9
<u>Aspergillus candidus</u>	11	8	9

<u>Capnosporella</u> spp.	11	4	9
<u>Trichosporon capitatum</u>	11	6	7
<u>Epicoecum purpurascens</u>	10	6	9
<u>Penicillium variabile</u>	10	6	8
<u>Rhizopus arrhizus</u>	10	7	6
<u>Torulopsis glabrata</u>	10	1	5
<u>Candida tropicalis</u>	9	6	8
<u>Penicillium cyclopium</u>	9	5	7
<u>Absidia ramosa</u>	8	4	5
<u>Aspergillus terreus</u>	8	4	5
<u>Candida</u> spp.	8	1	7
<u>Rhizopus</u> spp.	8	5	8
<u>Fusarium roseum</u>	7	5	6
<u>Mucor nr. saturninus</u>	7	2	6
<u>Penicillium aurantio-violaceum</u>	7	1	7
<u>Torulopsis inconspicua</u>	7	1	4
<u>Candida intermedia</u>	7	3	6
<u>Myrothecium verrucaria</u>	6	4	6
<u>Penicillium expansum</u>	6	3	6
<u>Penicillium herouii</u>	6	4	6
<u>Penicillium palitans</u>	6	3	5
<u>Penicillium ruberulum</u>	6	2	4
<u>Penicillium roquefortii</u>	6	2	5
<u>Prototheca</u> sp.	6	1	2
<u>Sporotrichum pruinosum</u>	6	2	6
<u>Coniothyrium fuckelii</u>	5	3	5
<u>Mucor globosus</u>	5	2	5
<u>Penicillium stipitatum</u>	5	4	5
<u>Penicillium stoloniferum</u>	5	1	4
<u>Candida humicola</u>	5	2	2
<u>Chaetomium globosum</u>	4	2	4
<u>Mucor racemosus</u>	4	3	4
<u>Cryptococcus laurentii</u>	4	3	4
<u>Penicillium charlesii</u>	4	3	3
<u>Penicillium fellutanum</u>	4	2	4
<u>Penicillium purpureogenum</u>	4	2	4
<u>Penicillium raistrickii</u>	4	3	3
<u>Penicillium simplicissimum</u>	4	1	4
<u>Penicillium velutinum</u>	4	2	4
<u>Stachybotrys atra</u>	4	4	4
<u>Syncephalastrum racemosum</u>	4	2	3
<u>Aspergillus</u> spp.	3	2	3
<u>Aspergillus ventii</u>	3	1	3
<u>Conidiobolus villosus</u>	3	1	3
<u>Fusarium</u> spp.	3	2	3
<u>Memnoniella echinata</u>	3	2	3
<u>Monilia sitophila</u>	3	2	3
<u>Mucor subtilissimus</u>	3	1	2
<u>Rhizopus rhizopodiformis</u>	3	3	2
<u>Torulopsis holmii</u>	3	1	3
<u>Acremonium</u> sp.	2	2	2
<u>Penicillium implicatum</u>	2	2	2
<u>Penicillium viridicatum</u>	2	2	2
<u>Rhizopus oryzae</u>	2	2	2
<u>Stilbellaceae</u>	2	2	2
<u>Trichosporon cutaneum</u> var. <u>multinormum</u>	2	2	2
<u>Zygothrychus welleri</u>	2	2	2
<u>Candida utilis</u>	2	2	2
<u>Penicillium spinulosum</u>	2	2	2
<u>Phialophora festiciata</u>	2	2	2

Table 38. p. 3

<u>Aspergillus clavatus</u>	2	1	2
<u>Circinella umbellata</u>	2	1	1
<u>Gliocladium catenulatum</u>	2	2	1
<u>Penicillium cascii</u>	2	1	2
<u>Penicillium clavigerum</u>	2	1	2
<u>Penicillium corylophilum</u>	2	1	2
<u>Penicillium rugulosum</u>	2	1	2
<u>Phycomycete</u>	2	1	1
<u>Torulopsis stellata</u>	2	1	2
<u>Trichothecium roseum</u>	2	1	2
<u>Yellow yeast</u>	2	1	2

The following species were observed only once, this appeared on only one date in only one location:

Absidia cylindrospora, Ascomycete, Aspergillus chevalieri, A. tamaris, A. ustus var. laevis, Candida curvata, C. pelliculosa, C. pulcherrima, Chaetomium sp., Coniothyrium sp., Curvularia interseminata, C. lunata, Cylindrocarpon radiculicola, Gilbertella persicaria, Gonatobotrys sp., Hansenula anomala, H. canadensis, H. ?suaveolens, Hemicola grisea, Kloeckera apiculata, Penicillium brevicompactum, P. cyaneofulvum, P. daleae, P. digitatum, P. duclauxii, P. italicum, P. lanosum, P. multicolor, P. pulvillorum, P. rolfsii, P. roseopurpureum, P. vermiculatum, P. waksmanii, Peyronellea sp., Pseudoplea americana, Pythium sp., Pyrenochaeta sp., Rhizopus microsporus, Rhodotorula minuta, Saccharomyces heterogenicus, Scopulariopsis brevicaulis, Sepedonium thielavioides, Smut-like, Sphaeronema sp., Sporidesmium bakeri, Sporotrichum olivaceum var. major, and Trichosporon margaritifera.

the flow of materials or as they were caught and held at one point or another in the treatment process.

On the basis of habitats in which fungi may remain and become active parts of the treatment process, the activities in the plant have been divided into four principal types. In the liquid system, which includes the liquid sewage as it passes from the raw influent through the preaeration tanks, through settling basins to the trickling filters, the final settling tanks and out to the Great Miami River, there is a gradual decrease in the number of colonies of fungi which are present. An occasional species may show an increase in numbers of colonies, but this may be related to the fact that on any one sampling date samples are obtained from different sewages as they pass through the plant. This is the phenomenon noted by Becker and Shaw (1955). However, reference to the other three types of stations which were sampled will indicate that, at least at Dayton, fungi were retained within one or another type of process, or, certain fungi found more favorable habitats for growth in one or another stage of the process.

In the solids system, in which sludges are digested, air dried, and treated for sale as soil conditioners, a somewhat different pattern of fungal development was found. Here, as sludges were held in the digestion tanks, and as oxygen supplies became more and more depleted, certain fungi appear to have produced considerable growth in spite of the fact that most of the molds listed are generally considered to be obligate aerobes. A few of the species listed, or their relatives, are known to be facultative anaerobes. Sludges on the drying beds included

good fungal populations as soon as they were poured on the beds. As these sludges lost their water through evaporation or transpiration, certain species increased in number, others died out. This finding could have been an apparent result of never sampling the same bed more than once. When heat was added to the treatment, many species of fungi died out probably as a result of being adversely affected by high temperature, others increased in number. Those increasing in number include mostly species which have thick, resistant spore walls, on which the effect of heat may have been to hasten germination. Their apparent increase may have been due to a revised ratio in their favor.

The permanent populations include the species which appear to have become adapted to life on the trickling filter. It has been shown (Cooke and Hirsch, 1958; see Section C) that of these, more than 20 species participate in the formation of the slime layer on the stone. This slime develops as the porous stone is colonized by organisms brought in with the sewage applied to the filters, or the air circulating over the filters. The growth of fungus filaments forms an interlacing network of tissue in which bacteria, yeasts and protozoans find refuge. Algal cells and filaments as well as filaments of such bacteria as Sphaerotilus, also cling to the stone of the filter bed and help make up the basic film on which such Metazoans as insect larvae, worms, and snails crawl and feed. Part of this film sloughs away as a result of various causes, but a basal layer remains attached to the stone and forms an inoculum which forms abundant new film quickly.

The fungi of the filter bed film appear to be aiding in purification of the sewage which passes over the film. None of the fungi found in sewage treatment processes have demonstrated their independence of other organisms for most, if not all, of their vitamin requirements. These fungi are able to use amino acids and ammonia as nitrogen sources, and only one could not use nitrate and nitrite nitrogen. Of simple to complex carbohydrate sources, most could not use lactose, and some could not use soluble starch. Some fungi which are present have been shown to use cellulose as a carbon source. Those species which were tested used this substance weakly in the laboratory. As settled sewage passes over the slimes on trickling filter stone, much of the solid material in it is adsorbed on the film and some of the dissolved and colloidal matter also may be adsorbed. The remainder of this material may be removed by organisms in direct contact with it by direct metabolic activity. The same group of species has also been observed in the liquid and solids systems.

At least five of the 27 habitats examined for fungi were materials destined for discard and incineration. However, it appeared to be of interest to determine the fungal content of these materials. Imhoff tank scum could not be sampled at all visits, because on at least one occasion there was none present. This condition was thought to have resulted from a heavy dose of detergents reaching the plant. The largest number of species of fungi found in the plant on any one sampling date was found in the Imhoff scum. FeCl_3 and lime are added to sludge in preparation for vacuum filtration. It was interesting to find that any organism could survive in materials treated this way.

C. Continuous Sampling of Trickling Filter Populations

1. Procedures*

The terminology of communities of organisms similar to those occurring on trickling filters has been referred to by Cooke (1956b) as: "the binding, free-living and grazing or scouring organisms on trickling filter stone form three interdependent communities within the periphyton of the aufwuchs biocoenose of the benthos, and they include both true benthic and facultative benthic-planktonic organisms which may be sessile or sessile-attached to the substratum. Those that are sessile-attached are true lithophytes, lithozoons or lithobionts, depending on the type of organism under consideration." In the same paper various methods were described for sampling such populations by exposing artificial bare areas for colonization.

Literature Review

Information on trickling filter populations is limited. Stanbridge (1954) (1955), in an extensive series of literature reviews on the development of biological filtration, has discussed the information available to him regarding the development of trickling filter films. These films include such organisms as algae, fungi, bacteria, protozoa, and metazoa. Cooke (1954b) has surveyed the reports dealing with fungi in such habitats. In his thesis on the fungi in two domestic sewage treatment plants, Becker (1953) also reviewed much of the literature in this field. Becker and Shaw (1955) observed fungi in progressively decreasing numbers in the effluents of successive processes in some sewage treatment plants. Filter bed slimes were sampled once; from the results of microscopic observations and platings it appeared unnecessary to sample this habitat further. Feldman (1955) has described several fungi in experimental filters.

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Tomlinson (1955) and Hawkes (1955) indicated that rate and periodicity of dosage have profound effects on filter populations. Less slime was produced on filters with long intervals between dosing than on those with short intervals.

In 1953, Heukelekian and Crosby (1953) were interested in the inhibition of various microorganisms forming slimes and causing deterioration of pipes, walls, and other structures for the conduction of waters, sewages, and industrial wastes. They found that slime accumulations were subject to retardation by sloughing; that the coatings studied were ineffective on either short- or long-term bases; that an initial retardation of growth was counteracted with time; and that a complete inhibitor in the concrete or the coatings was needed to prevent development of such growths.

In 1955 and 1956 Heukelekian and Crosby (1955a) (1955b) (1956) made a series of tests to determine the rate of slime accumulation on various surfaces under a variety of conditions in sewage-carrying channels and in the laboratory. In the laboratory, 3- by 1-in. glass slides were exposed to screened and settled sewage in submerged air-lift recirculation, alternate dipping, submerged dipping, and submerged static conditions. In addition, similar sized surfaces of transite and glazed tile were exposed. These materials were also used in studying the attachment of organisms in channels carrying sewage by exposing some 4-in. squares of the materials clipped to frames which were submerged for varying periods of time. The plates were submerged in a vertical plane parallel to the flow in the channel.

It was observed that the nature and texture of a surface had no ultimate

effect on the amount of slime growth, but initially smooth surfaces were less effective than rough. More growth occurred under aerobic than anaerobic conditions. The nature and concentration of food materials affected the production of slime: when food was reduced by artificial dilution or by removal and oxidation, growth increased; growth also increased when glucose and peptone were added. The velocity of the current increased the eventual amount of attached growth, but inhibited it during the primary period. The slime growth was cyclic: there was an initial lag, a rapid accumulation, a sudden decrease from sloughing followed by rapid accumulation, and more sloughing.

Moisture, ash, and nitrogen content of accumulated slimes corresponded to those of solids collected by settling raw sewage. It was considered that the 1 per cent nitrogen obtained from the growth was too low to be accounted for by cell substance alone, and, therefore, sewage solids were probably imbedded in the slimes. In considering the biological characteristics of the slimes, only bacteria and protozoans were emphasized and the occurrence of these was compared as to methods of slide exposure and the surfaces used. Flagellates were the most abundant organisms present in the anaerobic exposures whereas a wide variety of organisms was present under aerobic conditions. Protozoans were dominant in all types of submergence. This type of analysis indicated that only organisms recognizable to the observer through the microscope were considered important.

Various devices have been developed since 1915 for sampling selected portions of complex populations. In a technique described by Tomlinson (1941), five glass microscope slides 1 by 3 1/2 in. were mounted side by

side in photographic frames. These frames were laid on the bed of a stream and held fast by anchored brass chains. The results from four slides were examined quantitatively for accumulated weight; the fifth was used for identification and enumeration of organisms. Using this method, algae were studied in relation to stream pollution. Fourteen-day exposures were found to be inadequate for optimum results, but this technique was later modified for use in the study of populations on trickling filter beds. Four 3- by 1-in. microscope slides were mounted in photographic film holders, exposed on the surface of trickling filters, and the amount of growth was weighed periodically. The weights were found to be comparable to those of growth materials on similar areas of filter stones. The growth was found to vary quantitatively with 3- to 4-day and 7- to 14-day alternations in filter operation periods.

In studying the productivity of Sodom Lake, Mich., Newcombe (1950) exposed three series of slides during late summer and early fall to determine the "attachment material" "seeded on." One series of slides was used for determining the loss of weight on ignition, a second series for chemical studies, and a third series for a quantitative study of materials after suspending them in glycerin. The mean growth weights of the upper surface, the lower surface, and the combined surfaces of six slides were compared. It was found that the horizontal position gave best growths of attachment materials. From the results of this study, a formula was developed for "seeding-on" based on "compound interest at a constant rate."

Other techniques which have been developed for studies of this type rely on less critical quantitative or qualitative analyses. These consist of direct observation of natural materials attached to the slides, or of prepared slides. Using natural materials, specific groups of organisms only

are studied, ignoring any other types which may appear; with prepared slides, only specific groups for which special preparations have been made can be studied. Such exposures are also used to determine the efficacy of surface treatments to determine whether or not groups of organisms can survive under specified conditions. The basic technique for the present study was modified from one described by Tomlinson (1941).

Sample Collection

In a preliminary set of exposures, four glass microscope slides, 3 x 1 inches, were fixed in place in stainless steel photographic film holders with rustproof nichrome wire in such a way that a continuous surface was provided. Fifteen holders were placed on a low-rate trickling filter and 15 on a high-rate filter at the Dayton, Ohio sewage treatment plant. Pairs, one from each type of filter, were removed after 24 hours, 48 hours, 4 days, and 1 week, then at weekly or bi-weekly intervals until all were removed. The three-month observations proved inadequate because questions arose which could not be answered satisfactorily. The intervals between biweekly removals were too long to explain developing fluctuations.

The second series of observations was based on microscope slides fixed to pieces of 1/4-inch mesh galvanized hardware cloth 4 x 6 inches in size. The slides were mounted separately on the hardware cloth with 25- or 30-lb. nylon fishing line. Each slide was numbered, oven dried, and weighed before mounting. Each frame was identified by a numbered copper tag. Before exposure the completed frames were oven-dried and weighed. Sixty frames were placed in a row on a high-rate and a low-rate trickling filter on November 22, 1954. No attempt was made to place all the frames in the same position. Some slanted in one direction, some in another, a few were nearly horizontal, none were vertical, and most were at an angle of less than 30° from horizontal. One frame from each filter was removed after 24 hours, 48 hours, 4 days, and 1 week, and on each Monday thereafter. The last set was collected on December 26, 1955.

Sample Transportation

For purposes of shipping with minimum disturbance to the growth on the slides, a special wooden container was devised, with inside dimensions designed to accommodate the two frames after they had been wrapped separately in lead foil. The wrapped frames were then padded with cardboard to prevent movement within the box. Transportation in this was satisfactory. The growth, even after three or four days in transit, was still moist and typical; no sign of a septic condition appeared so that even Psychoda adults and larvae survived the journey in good condition. Frames transported to the laboratory by car were carried in a wire basket, protected from excess evaporation by wrapping the basket with a huck towel.

Laboratory Techniques

Upon receipt at the laboratory the frames were unwrapped and allowed to dry under room conditions for about 24 hours. This was sufficient time for the slides to become "air-dry" as evidenced by a tendency of the growth to flake. After drying the frames were weighed on a torsion balance. They were then dismantled by cutting the string and removing each slide carefully to a separate Petri dish. Each slide was then weighed. Excess materials on the frame were removed to separate Petri dishes and retained. The slides and excess materials were then stored in a refrigerator for further study.

Growth Removal

To enumerate and identify the populations of organisms on the slides, several techniques were tried. At first, the fungi formed the

focal point of this study but later, as algae increased in visible quantity, these also were quantitated. The numbers of bacteria, protozoans, and metazoans were not quantitated but these organisms could be seen in any fresh mounts made.

During the early stages of exposure, the slides were immersed in plates of dextrose phytone salts rose-bengal agar. Colonies developed around the periphery of the slides and over the exposed surfaces. Two slides from each frame were plated top side up, and two bottom side up, in order to get an estimate of the fungi occurring on all surfaces. As visible growth increased, after the first two or three weeks of exposure, various ways of removing the growth to determine its amount and type were explored.

At first, the dry slide was scraped with a scalpel. In this way much information about types of fungi and numbers of colonies was obtained, but on plating the slides it was found that much material remained, since about as many colonies remained on the slide as were removed. Finally, the slides were re-moistened and the growth and other materials removed with a rubber policeman. When this method was used to remove wet material, 5 to 15 percent of the organisms were left on the slide, as indicated by occasional slide platings. This percentage of residue was approximately proportional to the amount of material that had accumulated on and adhered to the slide.

To recover the dried growth in as short a time as possible, the slides were transferred from the refrigerator to a Petri dish and covered

with 20 ml of distilled water. The following morning the materials were removed from the slide with a rubber policeman. The materials from the top of the slide, as it was exposed, were kept separate from the bottom materials, as well as those excess materials associated with the slide but accumulated on the frame and enmeshed in the nylon string. Each of these three groups of materials was considered as "unit." No attempt was made to interpret "unit" in terms of weight of dry matter present.

Plating

Depending on the duration of the slide exposure, the top materials constituted 5 to 15 percent of the total weight of growth per slide, and the excess materials probably amounted to at least 200 percent of the weight of material on a single slide. Each unit of material was added to 80 ml of distilled water in a 250 ml Erlenmeyer flask. Materials from the top of the slide were then agitated in a Waring Blender at high speed for 10 seconds, bottom-of-the-slide materials were blended for 20 seconds, and excess materials for 30 seconds. These times gave relatively uniform suspensions for plating.

From these 1:10 dilutions, flask dilutions of 1:1,000, 1:10,000 or 1:50,000 were prepared. Later, when algae became evident on the slides, 20 ml of the 1:10 dilutions were removed for further study of types present, and algal cell or cell-clump counts were made. Each type of sample was inoculated in four replicate plates of dextrose phytone salts rose-bengal aureomycin agar as described above.

Incubation

The samples were incubated in room conditions of light and temperature for seven days, then counted and inventoried. With few exceptions, identification of colonies was made directly from the plates. A few colonies of special interest were picked for systematic studies at later dates. Most colonies not readily identifiable were lumped into general categories so that Moniliaceae spp., Dematiaceae spp., Penicillium spp., and "White Yeasts" were groups in which more than one species was represented. With the possible exception of the White Yeasts, the individual species forming these categories may be of little importance because of their small numbers.

Counting

The numbers of algae were determined by counting a portion of the sample after it had been fragmented in a Waring Blender. Samples from the tops or bottoms of the four slides in any one series were lumped together and counted in a Palmer nannoplankton slide at a magnification of 130 diameters. The numbers of fields counted ranged from 15 to 100 depending on the abundance of algae in the sample. The entire slide was then scanned to enumerate species not determined in the field count.

The first visible evidence of algal growth appeared on the slides on January 3, 1955. At that time the bottom surfaces of the slides from the high rate filter appeared green. The first slides examined quantitatively for algae were those returned on February 21, and the counting was continued to the end of the exposure period. Algae were counted as single cells in the case of unicellular species, or as clumps of cells in the case of colonial unicellular species and of those species whose

thalli were formed of simple or branched chains of cells. Because the growth mat was highly fragmented, it was sometimes difficult to determine the number of cells in a filament and whether or not the filament was branched. It was not practical to equate the cells of blue-green algae, green algae, and diatoms to produce a more accurate quantitative picture of the algae on the filters.

It is of interest to note that the Sladceks (1963) compared the technique of obtaining air-dry weights of slime reported above with a technique for obtaining oven-dry weights of accumulated slimes. They found the two methods to yield comparable results.

2. Populations*

Reports relative to sewage treatment contain few observations regarding the study of organisms inhabiting trickling filters. Available information pertinent to fungi in this habitat has been reviewed recently by Cooke(1954b)(1958a). The most important contributions to the knowledge of fungus populations have come from England (Tomlinson, 1946). Work in the United States has been restricted to studies of organisms which may have deleterious effects on the operation of filters (Cooke, 1954b), or to the commonest or most unusual species found on trickling filters in the treatment of industrial wastes (Hesseltine, 1953). Becker and Shaw found little or no fungus material in scrapings from filter stone on beds at Pullman, Wash., and Moscow, Idaho, and concluded that fungi were not of importance. The information on algal populations is even more scanty as a survey of the series of papers by Stanbridge (1955-6) shows.

Several species of fungi have been listed as occurring in the trickling filter population. These include: Fusarium aquaeductuum, Sepedonium sp., Ascoidea rubescens, Subbaromyces splendens, Geotrichum (usually listed as Oidium or Oospora lactis), Oospora fragrans, Penicillium sp., Distyachus sp., Pythium sp., and Sporotrichum sp. This list is based primarily on work done in England, New York, and New Jersey. Other studies on the biota of trickling filter slimes have not tried to identify "fungi" according to species. Various workers listing fungi occurring in filter slimes implied that a number of species may have been present.

Publications on the biology of trickling or percolating filters mentioned Chlorella, Stigeoclonium, Euglena, Phormidium, and Oscillatoria among the algae found in this habitat. In such publications no critical evaluation

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has been made concerning the species present. It was implied that other algae associated with polluted habitats occurred in trickling filters. Some of the problems in the study of such organisms have been discussed in relation to the study of bottom algae in streams.

Difficulties in evaluating numbers of bottom algae have been pointed out by Lund (1942) who stated:

There are, moreover, a number of factors mitigating against the accuracy and value of the counting method, particularly as applied to these types of flora. Thus the occurrence of some of the bottom-living algae in clumps or their movement into certain restricted temporarily favorable areas renders large-scale counting necessary to obtain correct numerical estimations.

Lund indicated that it was impossible to obtain counts large enough to achieve a degree of accuracy from the standpoint of time. A method was used which he recognized as inadequate but which was maintained for uniform results. In a preliminary survey of unknown habitats, he considered it of greater importance to obtain a general idea of the various types of communities and their variations, than to make single detailed observations.

Usinger and Needham (1954) cited a verbal communication by Margalef in which he stated that a comparative study of algae on glass slides and on the natural rocks in streams, indicated that only special kinds of algae attach to glass and that samples taken in this way are comparable neither in quality nor in quantity to samples taken under natural conditions.

Brooks (1954) set up laboratory cultures in large glass vessels containing samples of surface sand and algae taken respectively from a newly cleaned sand-bed and from a bed that had been in operation for 9 weeks.

Glass slides were placed on the sand and in the vessels, and weekly counts were made of the numbers of algae which settled on them, including both epiphytic and bottom-living forms. The counts showed that after a time the unattached algae disappeared from the slides in both vessels, although at first they appeared to grow well. Epiphytic forms such as Achnanthes, Ulvella, and Chaetopeltis, on the other hand, continued to thrive.

Blum (1954) placed sterilized rocks in unpolluted riffles of the Saline River in Michigan. Colonization by the diatoms Diatoma vulgare and Gomphonema olivaceum was so rapid that colonies became visible to the naked eye within 10 days. Colonization by Diatoma started October 27, and by Gomphonema about November 25. Colonization of Gomphonema ceased by April 10, indicating a seasonal capacity to colonize bare areas. It was concluded that both algae were capable of colonizing new areas at any time throughout their periods of growth and dominance.

General Populations

Each of the 20 filters at the Dayton sewage treatment plant supports a separate community or biocoenose of macro- and microorganisms. Each of these biocoenoses is independent of the other, except for common sources of inoculum and food materials. Filter sloughings, which become filter sludge, are settled out of the filter effluent in the final settling basin and returned to the preaeration chambers.

In starting a new filter, an inoculum from another filter may be used, or may be supplied from the settled sewage and the air. The length of time required for building up the biological film to an optimum association of organisms is uncertain. However, the length of time required for this association to effect an adequate removal of BOD, the index of efficiency of the filter, is known in many instances according to Gilcreas and Sanderson (1938). While it may take only 50 days for the appearance of the population characteristic of the mature filter, this does not mean that the population has reached a stable state in regard to all the conditions of its environment, both internal and external.

The surface film of the filters under consideration reached a maximum thickness of 2 to 3 mm during the 57 weeks of observation. This film was teeming with life of many types. It was continuously irrigated by spray from the nozzles of the rotary distributor. If the arms came to a stop or were slowed, the surface film dried and flaked off later with the rush of new flow, becoming part of the filter bed effluent.

The community of organisms comprising this film is built up without interference or disturbance from man, except for the manual removal of

undesirable elements, such as mosses which may grow around the edge of the filter (Cooke, 1954), flushing or drowning for control of the insect larvae whose adult stages become nuisances, or for major shut-downs.

The community of organisms on any one filter is made up of many types of microorganisms and a few macroscopic species. These include colonies and filaments of bacteria, colonies of yeasts, filaments of fungi, and colonies and filaments of algae. Moving about through this complex community are a number of kinds of protozoans (of which some may be stalked and thus sessile-attached), and nematodes. The larvae of several types of insects, as well as worms and snails which feed upon the slime, complete the population.

Fungi

In the filter beds the slime on the stone is formed by several phases of the complex community, depending on the depth of the filter. Only the surface phase has been examined in this study. The average number of fungus colonies observed per square centimeter of slide surface is shown in Figure 8. Below the first foot of the filter depth, where light does not penetrate but where aeration is maintained by the spacing of the stone, algae are probably absent or much reduced. The filter is aerobic with the possible exception of small areas which may become septic as excessive growth clogs the spaces between the stones. However, it is thought by some that septicity may develop at the base of the film, especially on the under side of the stones, and may contribute to the sloughing process.

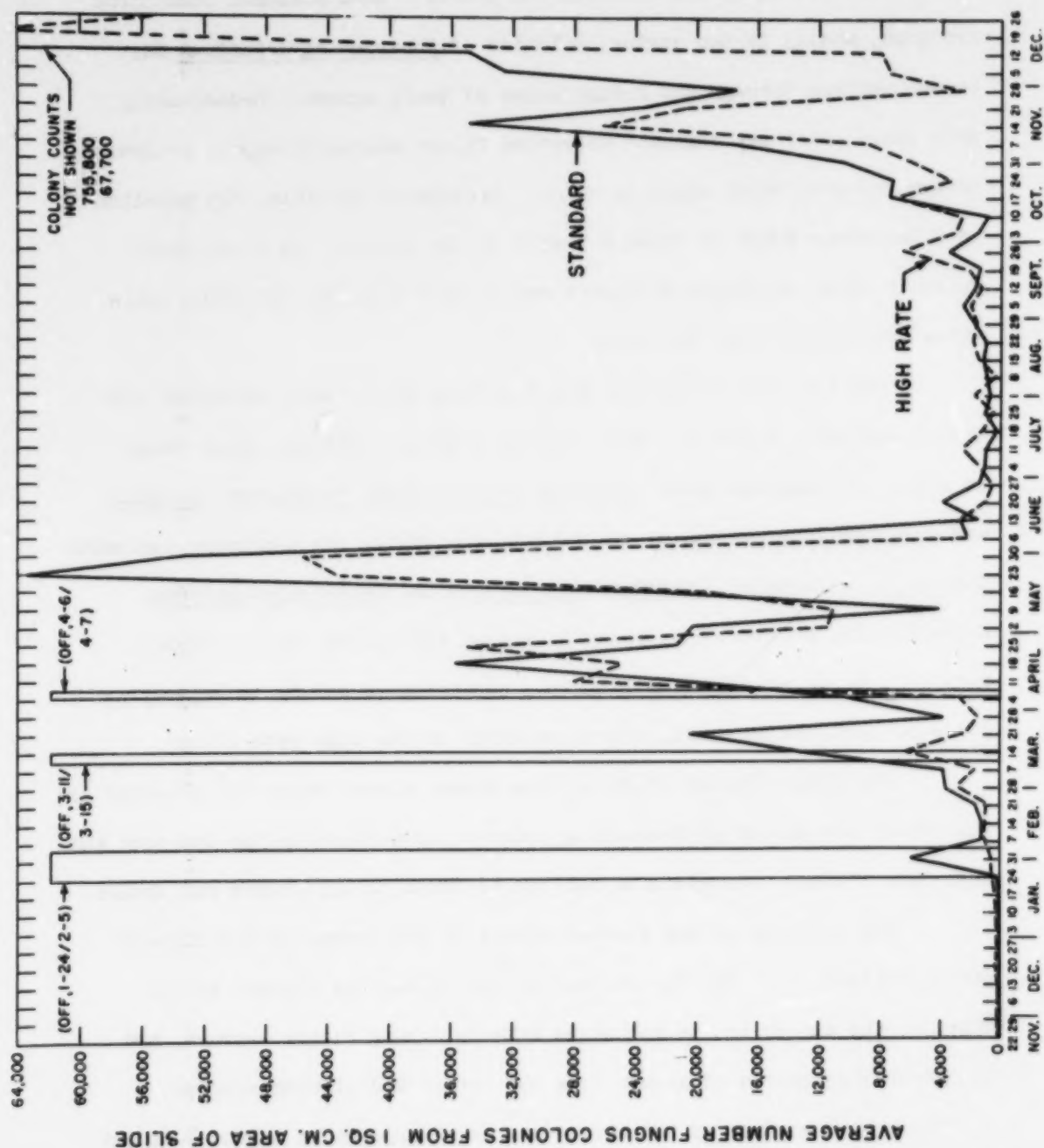


FIGURE 8

Average number of fungus colonies obtained from 1 sq. cm. of slide surface throughout the period of study.

The populations of filamentous fungi are usually as invisible on casual observation as the bacteria and yeasts. When seasonal conditions are good, mostly in the spring, colonies of Fusarium aqueductum may become obvious through the orange color of their spores. Occasionally this spore color may dominate an entire filter making it appear yellowish, orange, or even burnt umber in color. Throughout the film, the mycelium of filamentous fungi is found attached to the stones. On close observation a layer of fungus filaments can be seen covering the algal cells which intermingle with the fungi.

The low-rate filter and the high-rate filter each exhibited different dominant series of fungi. On the high-rate filter, these fungi in order of abundance were: Fusarium aqueductum, Geotrichum candidum, and Aureobasidium pullulans; on the low-rate filter the dominants included: Coniothyrium fuckelii, Fusarium aqueductum and Geotrichum candidum. Occasionally, in the cooler parts of autumn and spring, and in winter, mats of grey or white fungus material, appearing to belong to Leptomitium lacteus, occurred in small, scattered areas on the high-rate filter.

The fungi (Tables 39,40,41, and 42) and algae (Table 43) observed in this study are listed in descending order of occurrence on the low-rate and high-rate filters, according to habitat as found on the slides and frames.

The position of the exposed slides on the frames in the filters was as follows: 1. The top surface of the slides was exposed at all times to the sun or to its radiation effects during cloudy weather, and to direct application of sewage from the rotary distributor nozzles. 2. The bottom surface of the slides was never exposed to direct sunlight

Table 39. Occurrence of Fungi on Trickling Filters

Constancy Fungus Species Class	Per Cent Incidence in 57 samples				High Rate Filter			
	Low Rate Filter						Excess	
	Total	Excess	Bottom	Top	Total	Excess	Bottom	Top
I								
<i>Geotrichum candidum</i>	93.0	84.2	84.2	77.2	100.0	87.7	91.2	87.7
White Yeasts	96.5	86.0	93.0	84.2	100.0	91.2	96.5	86.0
<i>Fusarium aqueductum</i>	---	---	---	---	---	---	---	---
<i>Rhodotorula glutinis</i>	91.2	75.4	80.7	68.4	---	---	---	---
<i>Penicillium</i> spp.	98.3	82.5	77.2	70.2	93.0	84.2	82.5	66.7
<i>Gladosporium cladosporioides</i>	93.0	75.4	80.7	71.9	---	---	---	---
<i>Phialophora jeannelii</i>	93.0	71.9	64.9	56.1	---	---	---	---
<i>Coniothyrium fuckelii</i>	91.2	84.2	73.7	71.9	---	---	---	---
<i>Fusarium aqueductum</i>	89.5	80.7	86.0	66.7	---	---	---	---
<i>Alternaria tenuis</i>	87.7	64.9	60.0	66.7	---	---	---	---
<i>Aureobasidium pullulans</i>	82.5	71.9	64.9	43.9	87.7	71.9	68.4	59.6
<i>Penicillium</i> spp.	---	---	---	---	89.5	80.7	71.9	66.7
<i>Phialophora jeannelii</i>	---	---	---	---	87.7	71.9	59.6	59.6
<i>Gladosporium cladosporioides</i>	---	---	---	---	84.2	61.4	71.9	64.8
<i>Penicillium</i> spp.	70.2	43.9	60.0	49.1	63.2	36.8	54.4	54.4
<i>Trichoderma viride</i>	66.7	50.9	33.3	21.1	66.7	50.9	43.9	26.3
<i>Penicillium lilacinum</i>	61.4	42.1	49.1	28.1	68.4	50.9	54.3	38.6
<i>Alternaria tenuis</i>	---	---	---	---	73.7	45.6	35.1	59.6
<i>Coniothyrium fuckelii</i>	---	---	---	---	73.7	56.1	42.1	47.4
<i>Aspergillus niger</i>	---	---	---	---	59.6	26.3	40.4	14.0
<i>Aspergillus niger</i>	---	---	---	---	56.1	40.4	29.8	12.3
IV								
<i>Aspergillus niger</i>	42.1	29.8	15.5	3.5	---	---	---	---
<i>Aspergillus niger</i>	42.1	22.8	28.1	21.1	42.1	15.8	28.1	28.1
<i>Aspergillus niger</i>	36.8	27.3	7.0	8.8	---	---	---	---
<i>Aspergillus versicolor</i>	36.8	19.3	22.8	13.8	40.4	26.3	26.3	5.3
<i>Aspergillus fumigatus</i>	31.6	26.3	21.1	21.1	35.1	21.1	33.3	21.1
<i>Aspergillus ochrochloron</i>	24.6	17.5	12.3	8.8	29.8	17.5	12.3	8.8
<i>Aspergillus ochrochloron</i>	22.8	12.3	15.8	13.8	26.3	17.5	17.5	15.8
<i>Aspergillus ochrochloron</i>	22.8	14.0	5.3	1.8	19.3	7.0	14.0	5.3
<i>Aspergillus ochrochloron</i>	21.1	12.3	7.0	3.5	21.1	12.3	8.8	1.8
<i>Aspergillus ochrochloron</i>	21.1	10.5	13.8	---	22.8	17.5	14.0	3.5
<i>Aspergillus ochrochloron</i>	19.3	14.0	3.5	3.5	24.6	12.3	12.3	7.0
<i>Aspergillus ochrochloron</i>	19.3	10.5	7.0	3.5	31.6	14.0	14.0	10.5
<i>Aspergillus ochrochloron</i>	---	---	---	---	40.4	12.3	24.6	15.8

Table 40. Occurrence of Fungi on Trickleing Filters, Constancy Class V.

Fungus species	Per Cent Incidence	
	Low Rate Filter Total	High Rate Filter Total
<u>Fusarium oxysporum</u>	12.3	3.5
<u>Paecilomyces varioti</u>	10.5	12.3
<u>Aspergillus candidus</u>	10.5	8.8
<u>Subbaromyces splendens</u>	8.8	15.8
<u>Penicillium expansum</u>	8.8	7.0
<u>Gliocladium roseum</u>	7.0	3.5
<u>Aspergillus flavipes</u>	5.3	14.0
<u>Dematiaceae spp.</u>	3.5	12.3
<u>Monilia sitophila</u>	3.5	12.3
<u>Aspergillus spp.</u>	3.5	10.5
<u>Aspergillus sydowii</u>	3.5	10.5
<u>Penicillium herquei</u>	3.5	7.0
<u>Penicillium purpureogenum</u>	3.5	5.3
<u>Penicillium clavigerum</u>	3.5	3.5
<u>Chaetomium globosum</u>	3.5	1.8
<u>Fusarium spp.</u>	3.5	1.8
<u>Penicillium martensii</u>	3.5	1.8
<u>Stemphylium consortiale</u>	3.5	1.8
<u>Verticillium lateritium</u>	3.5	1.8
<u>Sporotrichum pruinatum</u>	1.8	5.3
<u>Verticillium spp.</u>	1.8	5.3
<u>Absidia spp.</u>	1.8	1.8
<u>Aspergillus ventii</u>	1.8	1.8
<u>Myrothecium verrucaria</u>	1.8	1.8
<u>Penicillium stipitatum</u>	1.8	1.8
<u>Penicillium variabile</u>	1.8	1.8
<u>Penicillium lanthipellum</u>	1.8	---
<u>Penicillium nigricans</u>	1.8	---
<u>Fusarium roseum</u>	1.8	---
<u>Mexoniella echinata</u>	1.8	---
<u>Mucor racemosus</u>	---	10.5
<u>Aspergillus clavatus</u>	---	7.0
<u>Gliomastix convoluta</u>	---	3.5
<u>Penicillium velutinum</u>	---	3.5
<u>Ascodesmis sphaerospore</u>	---	1.8
<u>Fusarium solani</u>	---	1.8
<u>Penicillium luteum</u>	---	1.8
<u>Stachybotrys atra</u>	---	1.8

Table 41. Populations of Trickling Filters

Dominance Fungus Species Class	Per Cent of Total Colonies				High Rate Filter			
	Low Rate Filter		Excess		Total		Excess	
	Total	Bottom	Top	Bottom	Total	Bottom	Top	Bottom
I								
<i>Coniothyrium fuckelii</i>	39.3	42.1	62.8	19.0	---	---	---	---
White Yeasts	17.0	15.9	8.6	25.7	27.9	23.5	16.2	27.1
<i>Geotrichum candidum</i>	14.9	13.3	3.0	22.7	23.1	21.6	4.2	27.9
<i>Fusarium aqueductum</i>	8.6	9.4	10.7	7.4	22.1	24.8	57.9	18.6
<i>Rhodotorula glutinis</i>	7.9	7.9	10.3	8.0	9.1	13.0	7.8	8.0
<i>Aureobasidium pullulans</i>	---	---	---	---	6.7	6.3	1.9	8.0
<i>Cladysporium cladosporioides</i>	2.7	3.2	2.4	4.3	3.9	3.7	2.8	4.5
<i>Aureobasidium pullulans</i>	3.3	3.6	0.6	3.9	---	---	---	---
Mon. liaceae spp.	1.8	1.3	0.5	1.3	1.7	2.2	1.9	1.7
<i>Penicillium</i> spp.	1.3	1.3	0.5	1.6	1.0	0.7	1.0	1.2
<i>Phialophora jeanselmei</i>	1.0	1.2	0.7	0.7	0.6	0.6	0.4	0.6
<i>Coniothyrium fuckelii</i>	---	---	---	---	0.9	1.4	1.9	0.6
<i>Penicillium lilacinum</i>	0.3	0.2	0.09	0.6	0.7	1.0	0.3	0.6
<i>Alternaria tenuis</i>	0.5	0.5	0.7	0.5	0.5	0.7	0.5	0.4
<i>Aspergillus fumigatus</i>	0.3	0.5	---	0.7	---	---	0.07	0.09
<i>Cephalosporium</i> spp.	0.2	0.1	---	0.4	0.2	0.3	0.2	0.1
<i>Trichoderma viride</i>	0.2	1.8	---	0.3	0.2	0.2	0.1	0.1
<i>Epilcoccus purpurascens</i>	0.1	0.2	---	0.1	---	---	---	---
<i>Penicillium funiculosum</i>	0.1	0.1	---	0.1	---	---	---	---
<i>Penicillium oxalicum</i>	0.1	0.1	---	0.2	0.1	0.1	0.2	0.1
<i>Aspergillus versicolor</i>	0.09	0.08	---	---	0.09	0.1	---	0.1
<i>Aspergillus niger</i>	---	---	---	---	0.2	0.1	---	0.1
<i>Penicillium ochrochloron</i>	---	---	---	---	0.2	0.4	0.1	0.2

Table 42. Populations of trickling filters. Dominance Class III.

Fungus species	Occurrence*	
	Low Rate Filter	High Rate Filter
<u>Absidia</u> spp.	*	*
<u>Aspergillus</u> <u>clavatus</u>	-	*
<u>Aspergillus</u> <u>niger</u>	*	-
<u>Aspergillus</u> <u>flavus</u>	*	*
<u>Aspergillus</u> <u>flavipes</u>	*	*
<u>Aspergillus</u> <u>ochraceus</u>	*	*
<u>Aspergillus</u> <u>sydowii</u>	*	*
<u>Aspergillus</u> <u>candidus</u>	*	*
<u>Aspergillus</u> <u>wentii</u>	*	*
<u>Aspergillus</u> spp.	*	*
<u>Ascodesmis</u> <u>sphaerospora</u>	-	*
<u>Dematiaceae</u> spp.	*	*
<u>Epicoccum</u> <u>purpurascens</u>	-	*
<u>Fusarium</u> spp.	*	*
<u>Fusarium</u> <u>roseum</u>	*	-
<u>Fusarium</u> <u>oxysporum</u>	*	*
<u>Fusarium</u> <u>solani</u>	-	*
<u>Chaetomium</u> <u>globosum</u>	*	-
<u>Gliocladium</u> <u>roseum</u>	-	*
<u>Gliomastix</u> <u>convoluta</u>	-	*
<u>Memnoniella</u> <u>echinata</u>	*	-
<u>Monilia</u> <u>sitophila</u>	*	*
<u>Mucor</u> <u>fragilis</u>	*	*
<u>Mucor</u> <u>plumbeus</u>	*	*
<u>Mucor</u> <u>racemosus</u>	-	*
<u>Myrothecium</u> <u>verrucaria</u>	*	*
<u>Paecilomyces</u> <u>varioti</u>	*	*
<u>Penicillium</u> <u>clavigerum</u>	*	*
<u>Penicillium</u> <u>expansum</u>	*	*
<u>Penicillium</u> <u>funiculosum</u>	-	*
<u>Penicillium</u> <u>ochrochloron</u>	*	-
<u>Penicillium</u> <u>martensii</u>	*	*
<u>Penicillium</u> <u>variabile</u>	*	*
<u>Penicillium</u> <u>purpureogenum</u>	*	*
<u>Penicillium</u> <u>herquei</u>	*	*
<u>Penicillium</u> <u>stipitatum</u>	*	*
<u>Penicillium</u> <u>nigricans</u>	*	-
<u>Penicillium</u> <u>velutinum</u>	-	*
<u>Penicillium</u> <u>luteum</u>	-	*
<u>Penicillium</u> <u>janthinellum</u>	*	-
<u>Phizopus</u> spp.	*	*
<u>Sporotrichum</u> <u>pruinsum</u>	*	*
<u>Stachybotrys</u> <u>atra</u>	-	*
<u>Stemphylium</u> <u>consortiale</u>	*	*
<u>Subbaromyces</u> <u>splendens</u>	*	*
<u>Verticillium</u> spp.	*	*
<u>Verticillium</u> <u>lateritium</u>	*	*

* The percent of total colonies represented by each species is less than 0.01 per cent in any category.

Table 43. Occurrence of Algae on Slides from Trickling Filters

Algae	Per Cent Incidence in 39 Sampling Periods			
	Position on Slide			
	High-Rate		Low Rate	
	Top	Bottom	Top	Bottom
Green Algae				
Chlorococcum-type	100.0	100.0	100.0	100.0
Stigeoclonium	61.5	61.5	15.4	46.2
Characium	-----	2.5	-----	-----
Blue-Green Algae				
Phormidium-Amphithrix	66.7	82.0	74.3	74.3
Pleurocapsa-type	0	0	61.5	35.9
Diatoms:				
Nitzschia palea	61.5	61.5	12.8	38.5

or radiation, nor to the direct application of sewage; it was protected from evaporation for longer periods when no sewage was being applied to the filters so it did not dry out as quickly nor as completely as the top surface. 3. Excess material, not necessarily directly associated with the slides, developed on the hardware cloth itself in the form of growth around the wires, growth on the marking tag and nylon string, entrapped sludges, etc.

Excess material, as well as materials from the top and bottom surfaces of the slides, was examined for fungi but not for algae. A layer of fungal, bacterial, and protozoan growth (apparently no algae in the early stages) covered the top side of the slide and screened the bottom growth from direct insolation.

The composition of the community on the trickling filters under study can be described best in terms of the ecological concepts of fidelity, constancy, and dominance. These three ecological terms may be defined as follows:

Fidelity is the degree to which a species is confined to a community or association.

Constancy, for the purpose of this discussion, may be defined as the frequency of occurrence in sample areas of uniform size exposed for cumulative regular periods throughout the sampling period.

Dominance is expressed by the estimated total count of colonies of fungi per unit or per square centimeter of slide surface.

Since the habitat under discussion is a rather restricted one, in which the organisms present are continually fed with a relatively dilute

aqueous nutrient, the fungi react differently than they do in the soil, the habitat with which most of these species are usually associated. In the soil these fungi remain dormant for long periods when no nutrients are available (Garrett, 1956). As nutrient-carrying substances such as pieces of leaves, sticks, and similar plant debris become available to the fungus, it develops rapidly, colonizes the new substratum, removes that portion of the material available to its enzyme system, sporulates abundantly, and again becomes quiescent.

In the trickling filter, nutrients are continually available and sporulation is reduced to a minimum in most species. It has been suggested (Cooke, 1957) that those fungi which have become adapted to the sewage habitat be considered coprophilous, but this term includes two of the four possible fidelity classes representative of this habitat - lyxobionts and lyxophiles. Lyxoxenes are present only on occasion and have not become adjusted to the habitat, and lyxophobes are never found in the habitat.

Except where special mention is warranted, data for the three types of habitat on the slides and sampling frames will not be considered separately when considering gross cumulative growth (Figures 9 and 10). When the slime is observed in place on the surface of the filter rocks, a layer of grey material covering the growth on the stones is apparent (Figure 11). When a stone is picked up, this grey film is broken, revealing the bright green color of the algae underneath. Next to the stones a thin to thick layer made up of fungal, bacterial and algal material in and among which the motile algae and protozoans carry on their browsing

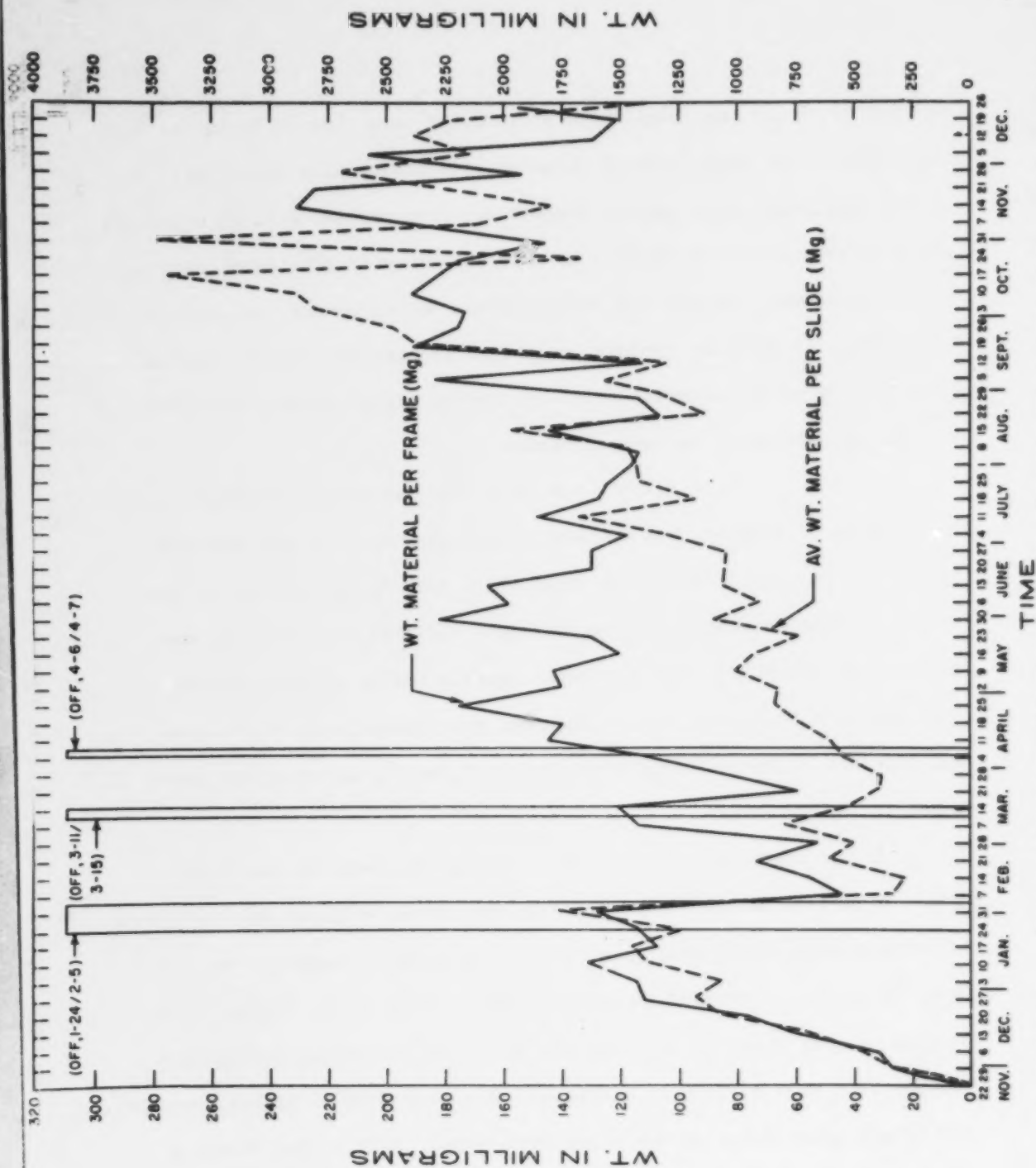


FIGURE 9 - CUMULATIVE GROWTH ON THE STANDARD-RATE TRICKLING FILTER.

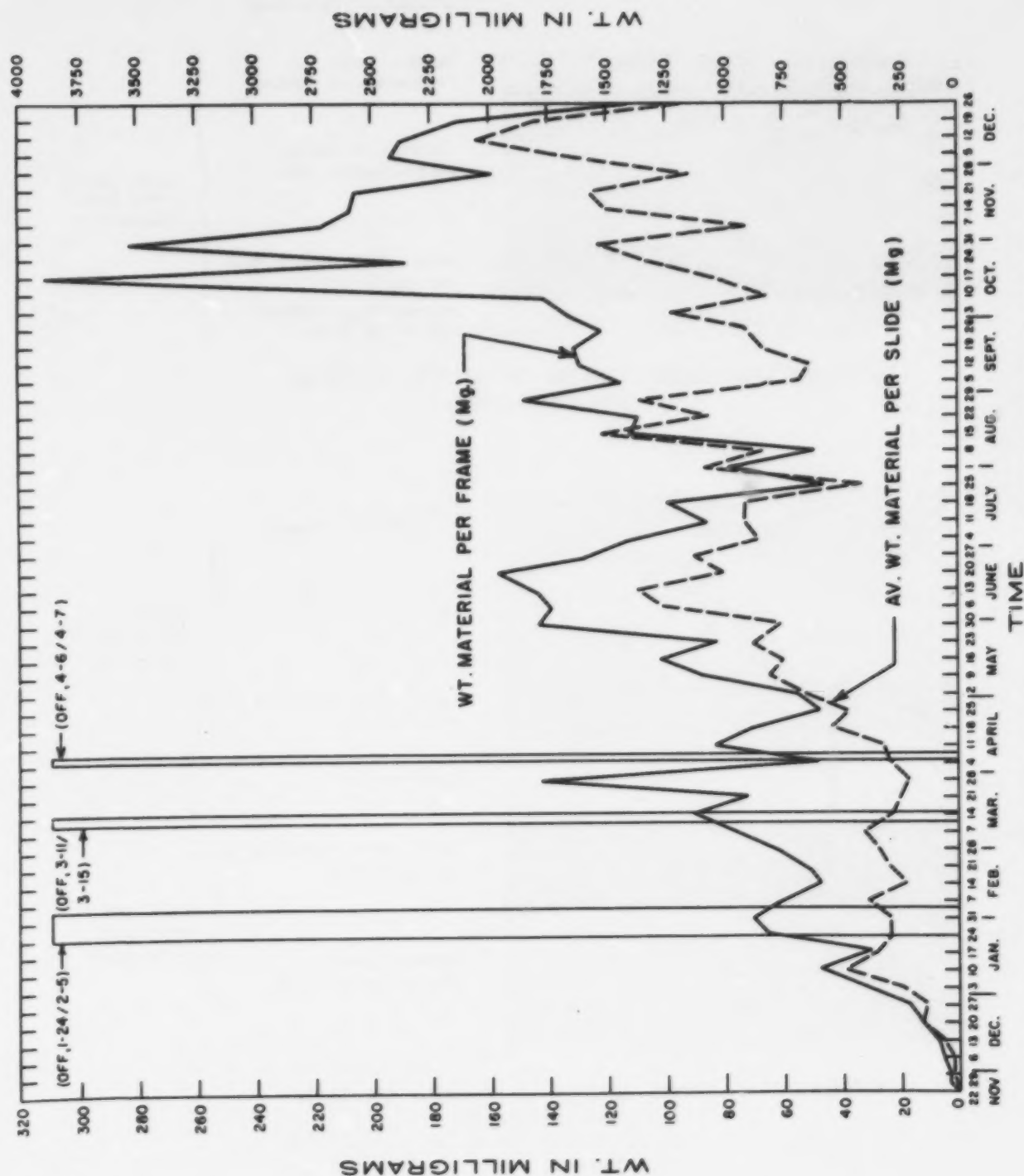


FIGURE 10 - CUMULATIVE GROWTH ON THE HIGH-RATE TRICKLING FILTER

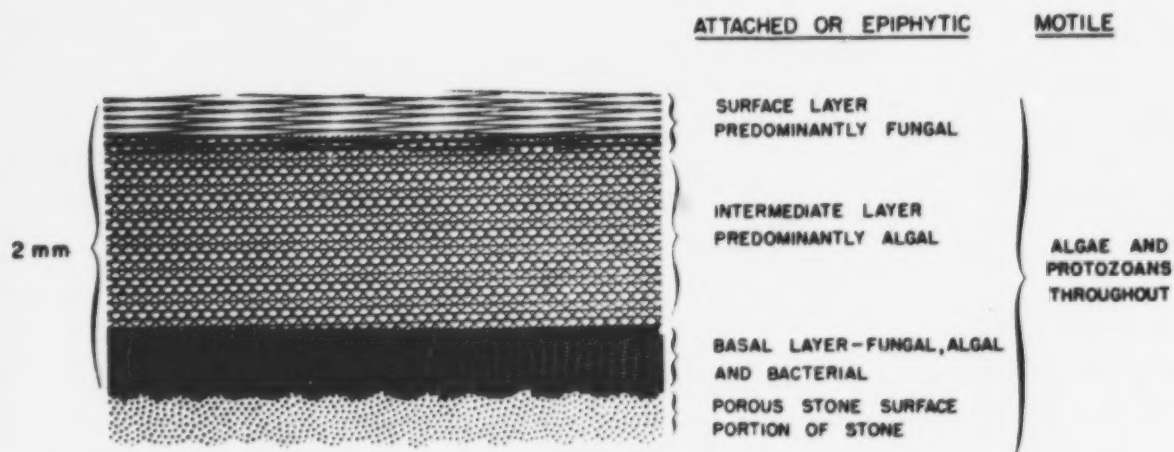


FIGURE 11a CROSS SECTION OF GROWTH ON STONE
(DIAGRAMMATIC)

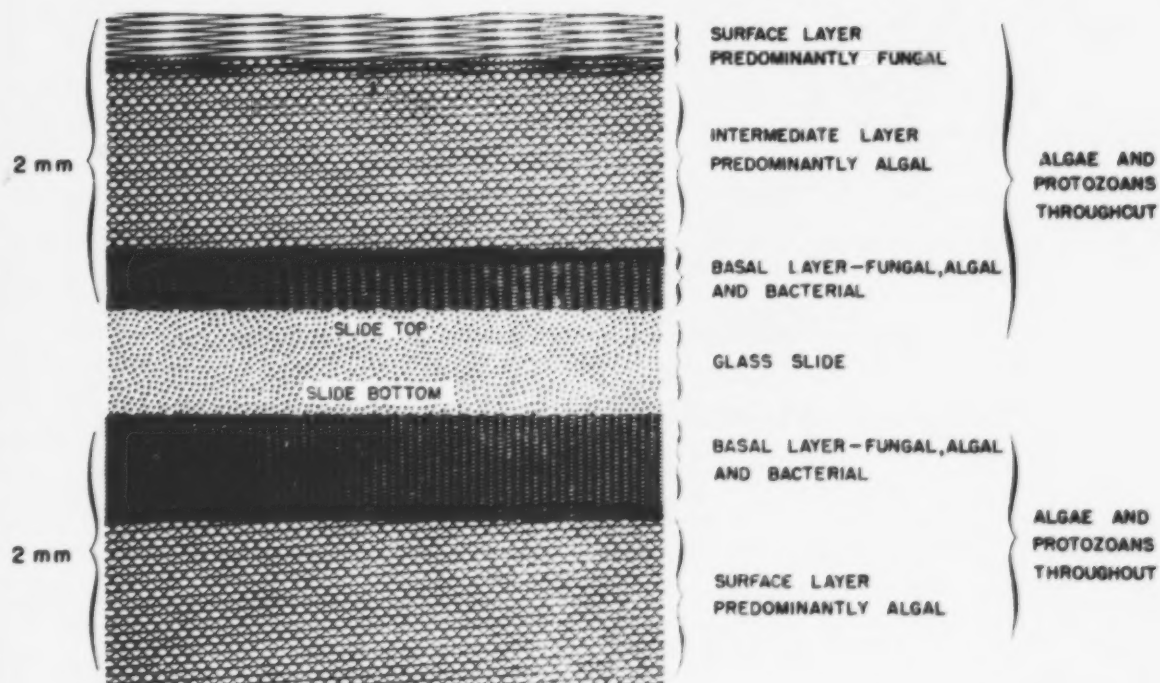


FIGURE 11b CROSS SECTION OF GROWTH ON SLIDE
(DIAGRAMMATIC)

or scouring action. It is suggested that the adherent material on both surfaces of the slides and on the surfaces of the rocks, is basically bacterial, fungal and algal.

On the upper surface, these organisms form an intertwined mass of which some members from each group are attached tightly to the glass. Apparently these slough off partially, but peel off completely only when dry. The whole mass is covered with fungal filaments. On the under surface the organisms adhere to the slide, with fungi and bacteria next to the slide. The fungi do not form a blanket over the algae, reversing the relationship on the rocks. Since the lichen-like layer of material on the rocks, composed of a layer of fungi, a layer of algal cells with intertwined fungus filaments, and a protective layer of fungi, has not been studied analytically as to the relationship of each organism or group of organisms to the whole, little more can be gained from a breakdown of these elements at this time.

The two communities under consideration, one on the high-rate filter, the other on the low-rate filter, are independent of each other from the point of view of space, even though the two types of filters are similarly constructed and are fed the same sewage, which is partially recirculated, the fungi and algae of both populations show a high degree of fidelity throughout the filter fields. From species lists published as a result of sampling other types of plants in New York, New Jersey, Massachusetts, Idaho and Washington, it may be said that there is a high degree of fidelity between the fungal populations of trickling filters in the United States.

Slides were removed from the stone of a high-rate and a low-rate trickling filter at weekly intervals for the 57-week period of observation. As a result of this study 5 constancy classes can be set up on the basis of percent of times observed. The species composing each class differ between the two types of filter as will be noted in Tables 39 and 40. If the three sampling areas of each slide are analyzed separately, each will give approximately the same analysis. However, it will be noted that certain species in Classes III and IV are not found as often on the slides as among the excess materials. Some others are found more often on the bottom of the slide, and still others on the top.

In tabulating the number of colonies of fungi found on plates poured from blended scrapings from top and bottom slide surfaces and from excess materials, it was found that three dominance classes could be established (Tables 41 and 42). Between the filters these are more consistent as to species composition than the five constancy classes. The third dominance class includes species represented by less than 0.01 percent of the total estimated colony count on the slides, although this count may number as many as 150,000 colonies or as few as 12 colonies. Here it is strikingly demonstrated that certain species, such as Coniothyrium fuckelii on the low-rate filter and Fusarium aquaeductuum on the high-rate filter, can tolerate the top surface of the slide almost to the exclusion of many other species present on the bottom surface and in the excess materials.

Species in constancy classes IV and V, and in dominance class III, include those found only once or twice up to 25 percent of the time. Most

of these species appeared as single colonies on only one of the four plates. Depending on the dilution of the sample used, this colony could be assumed to represent between 12 and 150,000 potential colonies, if the whole sample had been plated. This potential could be interpreted to mean that one colony was present on between 1 and 2,600 square cm of slide surface. On the other hand, a total of 118,000,000 colonies of Coniothyrium fuckelii was recorded on the low-rate filter exposures of which 19,000,000 colonies were on top and a like number on the bottom of the sampling slide. A complete list of fungal species observed on both filters during the 57 weeks of sampling is presented in Table 44.

Different Growth Habit Problems

Several factors must be considered in interpreting the enumeration of fungus colonies obtained from the slides. Bacteria, yeasts and coccoid algae produce cells in clumps. Such cells are not joined together in filaments, although in some cases cells are so arranged as to appear filamentous and may be enclosed in a mucilaginous sheath. On the other hand, the filamentous fungi and algae have cells which are arranged in filaments and the cells are not generally separable without rupturing the walls. In an aqueous habitat, such as found in trickling filters, most species of filamentous fungi usually do not produce spores, but in some cases atypical sporulation may accompany vegetative growth. In the case of the true yeasts, each vegetative cell may become a reproductive unit in much the same way that bacterial cells will function as propagules as they become dislodged from the colony and are carried by wind or water to new habitats.

Table 44. Species observed at Dayton during continuous sampling of surface populations of trickling filters.

Species	Number of Observations	
	High Rate Filter of 57	Standard Rate Filter of 57
<u>White Yeasts</u>	56	54
<u>Geotrichum candidum</u>	56	53
<u>Fusarium aquaeductuum</u>	56	52
<u>Penicillium</u> spp.	50	56
<u>Rhodotorula</u> spp.	53	52
<u>Phialophora jeanselmei</u>	50	53
<u>Cladosporium cladosporioides</u>	47	52
<u>Alternaria tenuis</u>	42	50
<u>Aureobasidium pullulans</u>	49	47
<u>Penicillium lilacinum</u>	39	35
<u>Trichoderma viride</u>	37	37
<u>Moniliaceae</u>	34	39
<u>Phoma</u> spp.	26	37
<u>Mucor fragilis</u>	31	22
<u>Epicoccum purpurascens</u>	24	25
<u>Aspergillus niger</u>	25	21
<u>Aspergillus versicolor</u>	23	21
<u>Aspergillus fumigatus</u>	22	15
<u>Cephalosporium</u> spp.	19	18
<u>Aspergillus flavus</u>	16	15
<u>Aspergillus ochraceus</u>	21	10
<u>Coniothyrium fuckelii</u>	17	12
<u>Penicillium oxalicum</u>	18	11
<u>Penicillium ochrochloron</u>	14	13
<u>Penicillium funiculosum</u>	14	11
<u>Mucor plumbeus</u>	13	11
<u>Aspergillus flavipes</u>	12	4
<u>Subbaromyces splendens</u>	9	4
<u>Paecilomyces varioti</u>	7	6
<u>Aspergillus candidus</u>	5	6
<u>Rhizopus arrhizus</u>	4	7
<u>Rhizopus rhizopodiformis</u>	9	2
<u>Dematiaceae</u> spp.	7	2
<u>Fusarium oxysporum</u>	2	7
<u>Monilia sitophila</u>	7	2
<u>Penicillium expansum</u>	4	5
<u>Aspergillus sydowii</u>	6	2
<u>Sphaeronema spinella</u>	4	4
<u>Aspergillus</u> spp.	5	2
<u>Mucor racemosus</u>	6	-
<u>Penicillium herquei</u>	4	1
<u>Gliocladium roseum</u>	2	2
<u>Penicillium clavigerum</u>	2	2
<u>Penicillium purpureogenum</u>	3	1
<u>Sporotrichum pruinosum</u>	3	1
<u>Aspergillus clavatus</u>	3	-
<u>Chaetomium globosum</u>	1	2
<u>Coniothyrium</u> sp.	1	2
<u>Penicillium martensii</u>	1	2
<u>Penicillium stipitatum</u>	2	1
<u>Stemphylium consortiale</u>	1	2
<u>Verticillium</u> spp.	2	1
<u>Verticillium lateritium</u>	1	2

Table 44. p. 2.

<u>Gliomastix convoluta</u>	2	-
<u>Myrothecium verrucaria</u>	1	1
<u>Penicillium variabile</u>	1	1
<u>Penicillium velutinum</u>	2	-
<u>Rhizopus sp.</u>	-	2
<u>Rhizopus nigricans</u>	-	2
<u>Absidia corymbifera</u>	-	1
<u>Absidia cylindrospora</u>	1	-
<u>Ascodesmis sphaerospora</u>	1	-
<u>Aspergillus ventii</u>	1	-
<u>Fusarium sp.</u>	-	1
<u>Fusarium roseum</u>	-	1
<u>Fusarium solani</u>	1	-
<u>Memmoniella echinata</u>	-	1
<u>Moniliaceae sp.</u>	-	1
<u>Moniliaceae sp.</u>	-	1
<u>Penicillium janthinellum</u>	-	1
<u>Penicillium ?luteum</u>	1	-
<u>Penicillium nigricans</u>	-	1
<u>Stachybotrys atra</u>	1	-
<u>Moniliaceae sp.</u>	*	
<u>Stemphylium botryosum</u>	*	

Several groups of fungi occur as intermediates between filamentous and yeast-like types. Among these are: (a) Geotrichum candidum, whose mycelium undergoes fragmentation, the individual cells becoming spores; (b) Aureobasidium pullulans, which produces masses of yeast-like cells from spicules scattered over the surface of the mycelium which themselves continue to produce yeast-like cells by a type of false-budding; and (c) Phialophora jeanselmei, whose spores may reproduce by budding and on whose mycelium abundant spores may be produced by spicule-formation. For filamentous stages in the development of these fungi and all other species of filamentous fungi, the blending technique of obtaining satisfactory inoculum for Petri dish cultures or pour plates gives a certain amount of inaccuracy in final counts. If the mycelial masses were broken up in an ideal manner, each alternate cell of a mycelium should theoretically give a colony and each interposed cell would be killed by the rupturing process. On the other hand, each yeast cell would produce a yeast colony, and in the case of those species which produce mycelium and yeast or yeast-like cells, both types of colonies could be produced.

Because of the possible loss of propagules in the blending procedure and in culturing, it is not expected that the plate counts represent the exact number of fungal cells in the original material. However, when the technique is repeated as exactly as possible, the results should be comparable and should indicate the relative importance of each species.

Because of the abundance of colonies and numbers and types of species obtained, it appears that most species on the filters react favorably to the culture procedure. The correlation between number of colonies and number of occurrences is another evidence that most species present react favorably to the treatment given before plating.

Possible Sources of Filter Populations.

In the activities of man, most of the fungi listed are simple contaminants. Many species are members of the normal populations of the soil and can reach the sewage treatment plant through runoff, drainage, the washing of dirty hands or soiled clothes. Many species can appear on decaying vegetable materials in the home. Mucor, Rhizopus, Penicillium, Aspergillus, and other common genera contain species which cause molds of bread, home-canned foods and unrefrigerated foods. They could have been introduced to the sewage treatment plant through disposal of spoiled foods.

In the food industry, many food materials are subject to decay by a wide variety of fungi whose spores are introduced during maturation, transit, or storage, and which may be encouraged in their activities under transit or storage conditions. Everyone is familiar with blue molds produced by species of Penicillium on apples and citrus fruits, or in the normal production of certain types of cheeses. Species of Alternaria, Rhizopus, Penicillium, Botrytis, and other fungus genera cause varying degrees of damage to most kinds of fleshy, juicy, and leafy vegetables and fruits. When such damage is noted in the home, in the market, in the warehouse, and even in transit, or on the farm or garden plot, the molded material is discarded.

Some of the fungi in the lists presented here are common contaminants of materials associated with the activities of man in the laboratory, hospitals and industry. One of the commoner contaminants of the dairy industry, from barn to dairy to home, is Geotrichum candidum. It is found in the dust of the barn, as a contaminant of cheese making, and is commonly present in old milk, cream, cottage cheese, and other milk products.

Whether or not it comes from these sources through sewage, it is one of the commoner fungi on both types of trickling filters where its white-grey mats may be observed when not filled with dark sludges or obscured by green algae.

Effect of Sloughing

During the course of time on the filter bed, growth may exceed the ability of the substratum to retain it, or excessive washing may remove it mechanically. When the flow to the filter is so low that the growth dries out, portions of it may flake off, and these flakes may be removed at the next application of sewage to the bed. This removal of growth contributes to the filter bed sludge which, after settling out in the final settling basin, is pumped as return sludge to the intake units. Thus, the organisms that grow on the filter bed stones become an additional source of inoculum for the preaeration and filter bed processes. Some of this sloughed growth may be carried in the plant effluent from the final settling basin to the river into which the effluent is drained.

Once a population of organisms has been built up on the stone of the trickling filter it appears to become a permanent part of the treatment plant. The stone of the beds under study was porous alag rock of uneven surface. The size and shape varied from 1 to 3 inches by 1 to 5 inches.

The entire population of a single stone never completely sloughs off; an inoculum of fungus, algal, and bacterial material clings to the

stone at all times. After severe sloughing this residual inoculum quickly rebuilds the population of the filter.

On the glass slides which were used as sampling devices, more than 100 mg (air-dry weight) of growth per slide appeared on both types of filter before flaking of the film. In flaking, some of the surface of the slide was left bare of any growth, and the slide appeared as shiny as when it was new. If the slide were wet before the flakes were removed, it was difficult to remove all the film, even from areas of this type, with a rubber scraper. Before sufficient growth developed to permit flaking, it was impossible to remove all the growth from the air-dried slide by scraping with a pocket knife or a scalpel, as shown by the production of colonies from slides which as been so scraped and then planted in a Petri dish with 20 ml of agar.

Since the slides constitute a smooth, relatively non-porous surface, it must be assumed that after sloughing or flaking from the filter stone a much larger inoculum remains because of its irregular surface and porosity. Depending on conditions, inoculum of resting cells could be held through periods of quiescence until the occurrence of favorable conditions of growth.

Algae

The numbers of algae were determined by counting a portion of the sample after it had been fragmented in the Waring Blendor. The samples from the tops or bottoms of the four slides in any one series were consolidated and counted in a Palmer nanoplankton slide (Palmer and Maloney, 1954), at a magnification of 430 diameters. The number of fields counted ranged from 15 to 100 depending on the abundance of algae in the sample. The entire slide

was then scanned to enumerate species not detected in the field count. Results of the counts are shown in Figure 12.

Throughout the exposure period, stones from both types of filters were returned to the laboratory several times for study of algae and possible culturing of species. All species enumerated were recorded for both slides and stones so that it may be considered that qualitatively the populations on the slides are typical of those on the filters. The species found were:

Diatoms

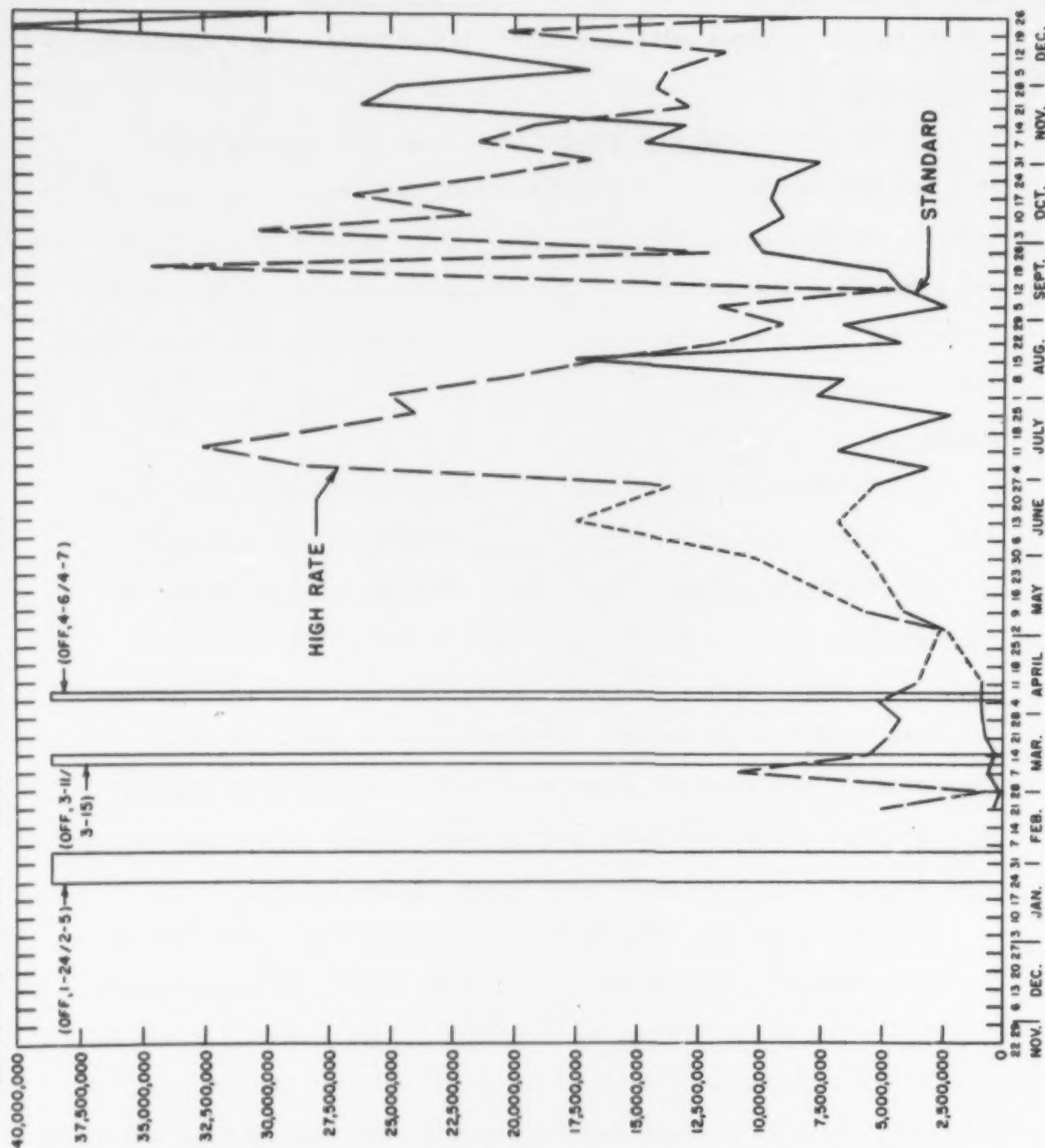
The only diatom found growing on the slides was a small and delicate form identified* as Nitzschia palea (Kutz.) W. Sm. It was suggested that the material could have been referred to N. kuetzingiana Hilse, which is very close to N. palea and of which it may be a variety.

Chlorophyta

1. Filamentous species. The two filamentous green algae found growing on the slides were identified¹ as Stigeoclonium nanum Kutz. and Ulothrix tenuissima Kutz. The Ulothrix could not be separated from the Stigeoclonium in counting because of the fragmented condition of the material. However, most of the material observed in the counts was definitely of Stigeoclonium. In one case, Characium sp. was observed growing attached to this alga.

2. Coccoid species. The coccoid green algae which formed a Chlorococ-cum-like growth on the filters could not be definitely identified, but it is believed to include a palmelloid stage of Stigeoclonium. Haensler, et al. (1923), reported that much of the green algae growing on a trickling filter at Plainfield, N. J., which they called a Protococcus stage, was reportedly observed developing into Stigeoclonium, but they could not state

NUMBER OF ALGAL CELLS AND CELL CLUSTERS PER SQ. CM. SURFACE OF SLIDES



TIME
FIGURE 12

whether or not all the coccoid cells belonged to the filamentous alga. According to G. W. Prescott, this group included algae identified as Chlorococcum humicola (Naeg.) Rab. and Chlorella vulgaris Beyerinck. Some of the palmelloid green cells were identified as "apparently a species of Chlamydomonas."

In addition, Scenedesmus bijuga (Turp.) Lag. and Oocystis parva West and West were found in cultured material, but these species were not observed in the material from the slides. It is possible that O. parva was present but unrecognized. On the rose bengal agar plates for fungus studies, Chlorella vulgaris formed occasional imbedded or superficial lens-shaped, dark green colonies.

Cyanophyta

1. Filamentous species. The two filamentous blue-green algae found growing on the slides were identified $\frac{1}{2}$ as Phormidium uncinatum (Ag.) Gom. and Amphithrix janthina (Mont.) Born. and Flah. The appearance of the Amphithrix was atypical, characteristic of the species in habitats where it is overgrown by other algae, as it often had curved and branched filaments instead of the upright, parallel filaments mentioned in descriptions of this species. Larger trichomes, which have been named var. torulosa (Grun.) Born. and Flah., were present, but the larger and smaller trichomes were considered as having a common origin.

2. Coccoid species. Most of the coccoid blue-green cells found on the slides from the standard-rate filter formed a Pleurocapsa-like growth of clumps of tightly packed cells. Anacystis montana (Lightf.) Drouet and Daily was of scattered occurrence on the slides. Drouet listed the Pleurocapsa-like growth as a parasitized form, possibly originating from A. montana, Entophysalis vulgaris (Kutz.) Drouet, or one of the Nostocaceae or Stigonemataceae.

* P. S. Conger, U. S. National Museum, Washington, D. C.

† G. W. Prescott, Michigan State University, East Lansing, Mich.

‡ F. Drouet, Chicago Museum of Natural History, Chicago, Ill.

(Now at: Academy of Natural Sciences, Philadelphia, Penn.)

Problems of Algal Counting

The accuracy of the estimates of algal numbers was limited by two general factors: (a) the limitations and errors involved in making the algal counts, and (b) the degree of uniformity of algae on the filters. The counting method presented several limitations in addition to the usual statistical errors involved in making plankton counts. The method of preparing samples, originally designed for enumeration of the fungi, had the disadvantage of making some algae difficult to recognize. Phormidium and Amphithrix had to be combined in counting because of this disadvantage. The Chlorococcum and Pleurocapsa types often appeared in clumps, and the number of individual cells in a clump was sometimes difficult to determine accurately. In the case of filamentous algae, each filament or fragment was counted as one unit. With Stigeoclonium, where fragments were much branched and widely varying in size, the numbers were only of general value as indicating the comparative abundance of this species from one group of slides to another.

Seasonal Appearance

Gross observations indicated that the most irregularly distributed growth was on the standard-rate filter, where the growth of Stigeoclonium, in particular, was spotty. This probably accounted for widely fluctuating numbers of Stigeoclonium in the counts. For example, on November 21, 1954, scattered patches of Stigeoclonium were observed on the standard-rate filter,

but none was detected on the slides collected on that date.

Although the filters appeared green from the beginning of the study, the first visible evidence of algal growth on the slides was observed on January 3, 1955, when the bottom surface of the slides from the high-rate filter showed green. Examination revealed the algae to be predominantly of the Chlorococcum type, together with a few Nitzschia cells. A few Chlorococcum-type cells were found on the tops of the slides from the standard-rate filter.

The first slides examined quantitatively were those collected on February 21, 1955. Scrapings from the rocks on the high-rate filter collected at that time were cultured in Gerloff's medium and growth of Chlorococcum-type and Amphithrix janthina was obtained. There are scattered records for Amphithrix on the slides from the high-rate filter from March through May, but some of these records are questionable, as they may represent misidentified fragments of fungus mycelium. Similar material was found on the standard-rate filter during that period, but it did not occur in sufficient numbers to be detected by the counting method. In later samples, Amphithrix was better developed and could be determined more definitely. Phormidium uncinatum was detected in abundance on the slides from both filters on May 30, and remained present for the duration of the study.

Stigeoclonium appeared on the filters during the summer months and, when well developed, covered the high-rate filter slides with a mat of green material. Tangled among the bases of the Stigeoclonium growths were clumps of Chlorococcum-type and large numbers of Nitzschia cells. Slides from standard-rate filters with well-developed growth had an entirely different

appearance. They were often covered with a flat brownish pad of Phormidium entangled with other algae. The appearance of the algal growths on the rocks of the two types of filters was somewhat different - dense green on the high-rate filter rocks, with a much spottier growth on the standard-rate filter rocks. An exception occurred in the center of the filter where continual leakage created high-rate filter conditions and the growth had the same dense green appearance as the high-rate filter growths. The growths were non-homogeneous, generally appearing in separate patches of the different types, and frequently overlapping. Except for the presence of Pleurocapsa-type cells on the standard-rate filter, the same species of algae were common on both types of filter. Differences in the algal biotas of the filters consisted in the relative importance and abundance of the various species.

Summary

The basic slime layer on trickling filter stones is composed of a complex of microscopic organisms including bacteria, fungi, algae, and protozoans. Of these, the fungi and algae appear to make up most of the basic attached materials with colonies of bacteria, fungi, algae, and

individuals of protozoans developing within or upon the basal mat. It appears inaccurate to refer to this complex as a "zoogloeal growth" (Usinger and Kellen, 1955) because it includes filamentous organisms as well as bacterial colonies of zoogloeal and other types. Most of the organisms present in the surface film on the top of the trickling filters studied appear to be capable of colonizing any surface offered, so that use of glass slides for their study seems feasible.

Members of the population of fungi studied could have been derived from local populations since they are common organisms in the environments that drain into the Dayton, Ohio sewage system. A few species of less common occurrence are more restricted in distribution or in habitat requirements. These fungi have become acclimated to the environment wherein they are continually bathed in a flow of sewage. Their oxygen requirements are satisfied by continual exposure to the air and their food requirements appear to be fully met by the nutrients occurring in the settled sewage and in the byproducts of the metabolic processes of other organisms in the filter.

Surface films on trickling filters or in areas of trickling filters receiving a continuous flow of sewage appear to have a definite structure (Figure 4). Underlying the mat and at least partially imbedded in the interstices of the porous slag rock is a layer of fungus mycelium, bacterial cells, algal cells or bases of filaments and holdfasts, and protozoa including sessile stalked species attached to the stone. From this fundamental layer arises a mass of tissue including fungal and algal filaments, clusters of bacterial cells, fungi, and algae, and free-swimming

protozoans and nematodes. Overlying this mass of material is another layer of fungal cells through which algae rarely penetrate, but in which yeasts, bacteria, and protozoans are found.

The sloughing process is still incompletely understood but is attributed to one or more of several phenomena including: (a) the grazing or scouring activities of insect larvae, snails and filter worms; (b) the occasional development of septicity in the lower portions of the film; (c) the mechanical action of sewage application after a period of quiescence; and (d) the development of excessive amounts of growth on the stones which cannot be held by the parental organisms at the base of the slime layer. As a result of observations made during the course of this study, the first and second hypotheses do not appear tenable for the filters under consideration. Following periods of excessive precipitation, when the filters receive an excessively high liquid loading, there appears to be more sloughing than at other times. Overproduction of slime components seems to play a part in sloughing toward the end of the summer season. Although the growth peaks show a semiannual pattern for fungi, this pattern is not reflected as sloughing in the over-all analysis of slide weights.

On trickling filters receiving a flow of sewage during only part of each day, a less clear pattern of slime development occurs. In many places the fundamental layer of fungi, bacteria and protozoans appears to be the only growth on the stones. Occasionally this layer is broken by spots of green algae where the pattern is much the same as that already described, or where small to large pads of blue-green algae occur as

separate colonies. The same type of colonies appear on the Glendale, Ohio filter during its periods of intermittent flow. Such pads are almost solid mats of algal filaments, and the arrangement of bacterial and protozoan growth within and around them is different than that for the typical filter slime.

Conclusions

The use of glass slides has been found to be an effective method of sampling the populations of microorganisms occurring on the surface of trickling filters. Isolation techniques of this study permitted the consideration of only fungi and algae, but it is recognized that bacteria and protozoans contribute important segments to the total population.

The mat on filter stones continuously receiving sewage appears to have a definite structure, while that on stones receiving an intermittent flow is more heterogeneous.

Sloughing is related more to the vigorous washing of excessive growth on the filters under consideration than to development of a septic layer at their base or to browsing or grazing of metazoal populations.

During a year the populations of the high-rate filter appear to be relatively uniform, although during the winter adverse conditions may reduce the algal populations and in the late winter and spring months Fusarium aquaeductuum may show marked increases. Standard-rate filter populations are governed by the intermittent flow of sewage and are much more erratic.

D. Trickling Filter Ecology *

Introduction

According to Clarke (1954), "the fundamental steps in the operation of an ecosystem are: (1) reception of energy; (2) production of organic material by producers; (3) consumption of this material by consumers and its further elaboration; (4) decomposition to inorganic compounds; and (5) transformation to forms suitable for the nutrition of the producers. If the area is occupied by a self-sufficient community, all these steps will go on within it".

On a large scale, and on the basis of a large population of microscopic or near-microscopic organisms, a trickling filter embodies many of the characteristics of the independent ecosystem as outlined above. However, the trickling filter is a man-made ecosystem and few man-made ecosystems are truly self-sufficient. The trickling filter is built for one purpose, the treatment of sewage by the action of a biota upon it. This biota is independent only as long as sewage is applied to the filter. The development of such a biota is relatively rapid. Its members are derived from the population of surrounding soils, waters, or habitats in which decaying or decomposable materials are present, as well as from sewage.

Basically, the trickling filter population is composed of decomposers, transformers, and locally, of producers which are partly dependent on the other members of the biota.

*Reproduced with permission from Ecology 40:273-291. 1959.

Habitat

In the United States today there are three principal methods of sewage disposal. One of the commonest is the direct release of polluted water into the nearest stream. A refinement of this process is found in the primary treatment of sewage in which the sewage is directed into settling basins with or without the addition of chemicals. After settling out of solid materials, the liquid is added to that of the nearest stream. However, purification (removal, by natural or artificial methods, of objectionable matter from water) usually results in only 25% - 40% BOD removal and approximately up to 70% suspended solids removal. The biochemical oxygen demand (BOD) of sewage is the quantity of dissolved oxygen, in mg/l, required during stabilization of the decomposable organic matter by aerobic biochemical action (Standard Methods, 1955). A further refinement is effected by any one of several biological treatment processes, commonly through the use of activated sludge or trickling filters. A sanitary engineer, whose duties include maintenance of a sewage treatment plant, thus becomes a biological engineer using tools of applied ecology.

A number of terms to be used in the description of trickling filters may be defined according to the "Glossary of Water and Sewage Control Engineering" (1949) for a clearer understanding of some of the problems to be discussed. In the following discussion, quoted terms and definitions are from this source. The broadest definition of sewage is "the water supply of a community after it has been fouled by various uses". This may be broken down into the two most important types of pollution, domestic or sanitary sewage and industrial wastes. The latter may be processed in

special treatment plants, or in conjunction with domestic sewage in municipal plants of the primary or secondary type. The municipal treatment plant operator usually is concerned with the former although he realizes that some industrial wastes are included.

The operation of a secondary sewage treatment plant is based on one or another method of "biochemical-", "biological-", or "sewage oxidation", a "process whereby, through the agency of living organisms in the presence of oxygen, the organic matter contained in sewage is converted into a more stable or a mineral form". Among the principal techniques of exposing sewage to the oxidative actions of microorganisms are the activated sludge process and the trickling filter. The latter is the only method with which we are concerned in this report. The anaerobic digestion of sludges is not considered here.

In the literature of sewage purification a number of terms have been used to describe the trickling filter. This structure is defined as follows: "A filter consisting of an artificial bed of coarse material, such as broken stone, clinkers, slate, slats or brush, over which sewage is distributed and applied in drops, films, or spray, from troughs, drippers, moving distributors, or fixed nozzles, and through which it trickles to the underdrains, giving opportunity for the formation of zoogloal slimes which clarify and oxidize the sewage." The process is termed "biological filtration." The structures have been termed "bacteria beds," "contact beds", and "percolating filters". The modification in which the spray comes from fixed nozzles has been termed a "sprinkling filter." Depending on the rate sewage is applied to the filter after primary settling, the filter may be

termed "high-rate" (dosed at 10-30 million gallons per acre per day - mgad), or "standard-rate" or "low-rate" (dosed at 1-4 mgad). Since the Orford Subcommittee (1957) prefers the use of "low-rate" to "standard-rate", this term is used in the following discussion. Other modifications of the trickling filter are in use, but the work on which this report is partly based has been done on low-rate and high-rate trickling filters.

As indicated above, the trickling filter exists to support a biological community referred to as a "zoogloal slime". This has been referred to commonly as a "microbiological film" which is defined as the "gelatinous film of zooglian growths covering the media or spanning the interstices of a biological bed". "Zoogloea", in the terminology of sewage treatment, is defined as a "jelly-like matrix developed by bacteria. The word is usually associated with activated sludge growths in biological beds". The bacteriologist is more specific in his use of the term. To him Zoogloea is a distinct genus of bacteria, as well as the type of bacterial growth produced during certain phases of the life cycle of single, colonial, or filamentous bacteria which can produce gelatinous matrices.

Finally, the term "degree of purification" may be mentioned. This phrase is defined as "a measure of the removal, oxidation, or destruction of solids, organic matter, bacteria or other specified substance affected by sewage treatment processes". The degree of purification of sewage represents the efficiency of the sewage treatment plant. This rating can be used as a means of obtaining more funds with which to build a more efficient plant, or as a means of evaluating the residual waste products of a community which are being emptied into streams. The purity of such water is important to other communities downstream and to the biota of the stream.

The trickling filters studied most intensively by the writer are those in the Dayton, Ohio sewage treatment plant. This plant is primarily concerned with the treatment of domestic sewage. The conditions of the influent are relatively constant since only rarely do loads of industrial wastes exceed the capacity of the plant to handle them with the domestic sewage. Eight of the 20 filters are of the high-rate type and are dosed continuously throughout the year. The remaining 12 filters are of the low-rate type and receive the excess portion of the settled sewage. Their dosage is, therefore, essentially intermittent, occurring only during periods of the day when the plant is receiving peak loads. Living conditions for the biota of the low-rate filters are different from those of the high-rate filters. Data on effectivity of operation of the two types are not available since effluents are composited before analysis.

The filters are 165 feet in diameter, 7.5 feet deep, constructed in the form of a concrete cylinder with a funnel-shaped channeled base, and are filled with more than 160,270 cubic feet of limestone slag. On the porous surface of this rock a population of microorganisms has developed. Because it is impractical to dig deeply into this mass of rock, which varies in size from two to five inches in diameter, only the surface layer of stone has been examined periodically and regularly.

Since the habitat is man-made, and since it was made for a specific purpose, it is reasonable to expect that conditions in the habitat will be modified from time to time to increase the efficiency of the structure and its population. The most noticeable effects on the filter biota are

produced by altering of dosing rates, when the filters are shut down for repairs, or when excessive runoff from precipitation dilutes the sewage.

Effects of changeable air temperature on the filter surfaces are ameliorated by the relatively constant temperature of the continuous dosage of settled sewage.

Relatively little ponding occurs in the filters. Spaces between the stones are large enough in most cases so that cross growth has not occurred and the filters are well ventilated. Conditions of excessive speticity rarely, if ever, develop.

Biotic effects of the population appear to be relatively constant. If disseminules of additional species reach the site and the species becomes effective in the population, little change will result since the new species will have replaced a species unable to maintain the competitive level. However, it is not to be implied that numbers of species are fixed. Effects of various groups of species in the population are relatively constant since they are apparently controlled by interaction and available food supplies. A possible exception could be found in seasonal over-population of snails or worms neither of whose influence is reflected in the activities recorded in the wiegths of material found on sampling slides, or in species or colonies of organisms isolated therefrom.

Populations

Introduction

The biotic populations of the trickling filter are relatively uniform in composition. That is, bacteria, fungi, protozoans, algae, nematodes, rotifers, snails, sludge worms, and larvae of certain insects of which the foregoing are composed, may vary from filter to filter, at the species level, but the biota, whether the filter is in the United States or in Europe, remains relatively constant. Occasionally, when conditions permit, other organisms may also be found such as the mosses reported at Dayton (Cooke, 1953). Such organisms are accidental invaders, are restricted to marginal areas, and rarely complete their life cycle, remaining in the vegetative state.

In the past identification of the members of this population has been of little concern to the sanitary engineer as long as the population became established and functional relatively quickly after placing a filter in operation. A good operating condition is one in which little clogging occurs in the spaces between stones and one which carries on its function of removing solid, colloidal or dissolved materials from the sewage, thus reducing its biochemical oxygen demand.

The presence of bacteria has been considered normal. The species which are present have not been evaluated beyond the requirement that the coliform content of the final sewage discharged into the receiving stream be low or lacking. Fungi have been considered nuisances more than aids in the biological treatment of sewage (Becker, 1954; Cooke, 1954). Tomlinson in England (1941, 1946) has found that several species of fungi

are members of the population, as have Hesseltine (1953) and Cooke (1957). On the other hand, Becker and Shaw (1955) report that the occurrence of fungi is seasonal and of little significance. Lackey (1924) has surveyed the protozoan populations in New Jersey while intensive population studies have been carried on with these organisms in England. The occurrence of algae has been noted occasionally according to a survey of Stanbridge (1955). His finding seems odd since algae are invariably present on the surface of trickling filter rock in the many filters which have been observed by the writer. Insect populations have been studied in England relatively intensively (Hawkes, 1952), because certain species have become nuisance problems in that country. Discovery of control methods has appeared to be important. Recently problems of snail control have been considered by Ingram et al. (1958) in connection with equipment damage and nuisance problems.

Composition of Microbial Film

To date, few comprehensive studies have been published in which the biota of the zoogloeal slime has been described. Bacteriologists, mycologists, algologists, entomologists, or protozoologists have been interested in one filter or another, at the surface or more rarely at several depth levels, and have reported on their observations without overlapping into the interrelated fields. Occasionally a few organisms from one or more of the other members of a population have come to the attention of the observer but the resulting lists are incomplete. Consequently, a composite list of organisms is presented to indicate the

complexity of the populations on the trickling filter. It would be difficult for one individual to study the whole population and its inter-relationships comprehensively since the organisms involved cover such a wide range of groups of species in plant and animal kingdoms.

A report by Heukelekian and Crosby (1956) includes relatively representative chemical and physical data on the composition of zoogloal slimes. Since it is difficult to get materials by direct scrapings from trickling filter stone, unglazed tiles were exposed in channels carrying sewage to obtain slime growths of similar composition to those found on trickling filter stones. Table 45 indicates the type of physical and chemical data of interest to the sewage treatment plant operator.

In studying the populations of the sewage treatment plant and its included processes, the bacteriologist is interested primarily in the occurrence of coliform bacteria. This group has long comprised one of the most important groups of organisms indicative of sewage pollution (Kabler, 1957). High coliform density indicates a high degree of pollution. One requirement of effective sewage purification in some communities is the removal of as many coliform bacteria as possible. Thus, beyond routine checks of m.p.n. (most probable number) of coliform bacteria, whether done by the Standard Methods (1955) or the membrane filter (Clark et al., 1951) technique, little attention is paid at present to other kinds of bacteria present on trickling filters. No comprehensive species lists have been found in the literature for this habitat. However, Hotchkiss (1924) prepared a list of bacteria by their activities on sprinkling filter beds in New Jersey. An average of 20-24 samples obtained

Table 45. Analysis of slimes (after Heukelekian and Crosby, 1956).

Determination	Results
pH	8.6
Density at 20°C	1.0122
Moisture, % of wet weight	95.4
Total Solids, % of wet weight	4.6
Volatile solids, % of total solids	70.7
Ash, % of total solids	29.3
Total nitrogen, % of total solids	1.0*
Ammonia nitrogen, % of total solids	0.5
Grease, p.p.m.	15.7

* This determination is low for a mass of organisms, but materials on which the determination was based include sludges and other inert matter which would tend to reduce the ratio of nitrogen to other elements present.

throughout the year gave the following numbers of bacteria in thousands per cc of filter slime: nitrate reducers 208.4, H_2S producers (from protein) 172.9, albumin digesters 102.6, species which oxidize nitrites to nitrates 53.3, species which oxidize NH_4 salts to nitrates 39.8, H_2S producers (from sulfates) 19.9, and species which reduce thiosulfates to sulfate 2.0.

As early as 1914, Johnson, in England, observed bacteria and fungi on filters. He considered that Sphaerotilus natans was among the important bacteria on the beds he studied and that it took the form of Zoogloea ramigera and its varieties compacta, carnea, and uva under certain circumstances of growth. The review of the literature on the fungi of sewage by Cooke (1954) indicated that Sphaerotilus natans is widespread in sewage and polluted water, that it is known in several forms referred to in the literature as species and even genera, and that it can assume a zoogloea stage in its life history. Johnson reported on the appearance of seven fungi on trickling filters, but the nomenclature he used, while probably valid at that time, refers now to such different organisms, or organisms listed today under such different names, that to repeat them would only add to confusion.

In studies on the fungus populations of trickling filters, both in connection with the technique of sampling such habitats with glass slides (Cooke, 1958) and with sampling the populations by scraping the slime from stones (Cooke and Hirsch, 1958), 90 species of fungi have been presented. Of these, more than 20 species are considered to be members of the permanent population of fungi found on trickling filters.

During much of the year the trickling filter is covered with a green film of algae. This is only one of the several types of organisms in the slime on the upper or surface layers of stone. Except for those algae which are washed down through the filter and somehow manage to survive, these organisms are limited in distribution to those portions of the filter exposed to the light. Early listings included the genera Chlorella, Stigeoclonium, Euglena, Phormidium, and Oscillatoria without regard to the species represented. In a recent paper by Cooke and Hirsch (1958), the following species were listed from the Dayton, Ohio trickling filters: Nitzschia palea, Stigeoclonium nanum, Ulothrix tenuissima, Characium sp., Chlorococcum humicola, Chlorella vulgaris, Phormidium uncinatum, Amphitrix janthina, Anacystis montana, and Pleurocapsa sp.

It was found that high-rate and low-rate trickling filters supported different populations of algae especially when loadings were distributed over different periods of time. At Dayton, the high-rate filters receive settled sewage continuously throughout the 24 hours of the day while the low-rate filters receive only that in excess of the high-rate filter capacity. Where leakage occurred around the base of the column supporting the distributor arms, the pattern of slime growth is similar on both filters. Diatoms and green algae predominated on the high-rate filters, while blue-green algae were abundant on the low-rate filters.

Extensive work on the protozoans and other animal life in the sprinkling filter beds at Plainfield, New Jersey was published by Lackey (1926). During the course of his study it was found that 19 rhizopods (Sarcodina) occurred in the microbial film on the filter stones. Stones from 1-, 3-,

and 5-foot depths were examined and the commoner species quantitated. The following rhizopod species were listed: Actinophrys sol, Actinosphaerium eichhornii, Amoeba proteus, A. radiosa, A. verrucosa, Arcella dentata, A. vulgaris, Centropysis aculeata, Chlamdophrys stercorea, Cochliopodium bilimbosum, Diffugia pyriformis, Euglypha alveolata, Hartmanella hyalina, Pamphagus nutabilis, Protamoeba primitiva, Trinema lineara, Vahlkampfia albida, V. guttula, and V. limax. Lackey's earlier paper on filter bed organisms in New Jersey (1924) listed the following genera of rhizopods as including commoner species of: Amoeba, Arcella, Diffugia, Euglypha, and Trinema.

In addition to work on rhizopods, Lackey (1924) listed the various genera of flagellates and ciliates which occurred on New Jersey filters. In addition to "small flagellates" in the Mastigophora, the genera Distigma, Euglena, Heteronema, and Notoselenus were listed as including at least one species each. In most work with rhizopods, flagellates, and ciliates in the United States the organisms are listed only according to genus. Such listing indicates a lack of interest in identification below the generic level, or difficulties in making specific identifications which would require more time than is available for this type of study.

The genera of ciliates (Infusoria) as listed by Lackey (1924) included: Opercularia, Aspidisca, Blepharisma, Chilodon, Colpidium, Frontonia, Lembus, Lionotus, Metopus, Oxytrichia, Paramoecium, Pleuronema, and Vorticella. In 1926, Lackey added to this list some genera of less common occurrence: Prorodon, Macrystaria, Holophrya, Nassula, Chilodon, Loxodes, Loxophyllum, Amphileptus, Lionotus, Lionotopsis, Glaucocma, Uronema,

Colpoda, Colpidium, Microthorax, Lembus, Cyclidium, Pleuronema, Paramoecium, Stentor, Blepharisma, Metopus, Spirostomum, Uroleptus, Amphisia, Oxytrichia, Stylonichia, Euplotes, Aspidisca, Opercularia, Vorticella, and Carchesium. In the Suctorina, only the genus Podophrya was listed. Butcher (1932), in England, has indicated the importance of Carchesium, Vorticella, and other stalked ciliates in the biota of sewage by listing them among the organisms called "sewage fungus". Butterfield, Purdy and Therriault (1931) advanced a theory of the place of these organisms in sewage treatment following a series of experiments at Cincinnati. They stated that "the chief function of certain plankton (Colpidium, a ciliate) in the biochemical oxidation process is to keep the bacterial population reduced below the saturation point and thus to provide conditions suitable for continuous bacterial multiplication, thus in turn resulting in more complete oxidation". Therefore, certain protozoans are seen in both activated sludge and trickling filter slime as browsing or grazing organisms which may remove some solid, colloidal, or dissolved organic materials, but which primarily aid in the control of the bacterial populations. In some cases, yeast cells may be ingested also but the protozoan organisms are usually too small for effective control of the yeast populations.

In a study of the biology of sewage in the Brent and East Ham sewage treatment plants in England, Peters (1930a) listed the protozoans and other animals found throughout the treatment plants. He did not indicate which organisms were found in the trickling filters although 39 of the 52 categories listed were found in them.

Peters (1930b) was especially interested in the nematodes occurring in sewage treatment plants in England. The species Diplogaster strictus, Diploscapter coronata, Dorylaimus saprophilus, and Rhabdites sp. were listed. Workers in the United States, such as Lackey (1924) and Wilson (1943), were content to list nematodes as a group category without attempting to resolve the populations into the various genera and species which were present. These are among the larger microscopic animals in trickling filter film and any mount will include some individuals.

Among other members of the animal populations of the sprinkling filters at Plainfield, New Jersey, Hausman (1923) listed unidentified nematodes, rotifers, annelid worms including Aeolosoma and Pristina, and Tartigrada. Combined counts at three levels in thousands per cc of centrifuged film showed some seasonal variation in the occurrence of these animals.

In 1939, Reynoldson made a very thorough study of (enchytraeid) segmented worms in bacteria beds in England. The most important species was considered to be Lumbricillus lineatus while Lumbricus rubellus was also abundant. Usually adults were found at lower levels while the young were near the surface. Their feeding habits were closely associated with the growth of Phormidium sp. on the surface film. Drying conditions in summer and cold temperatures in winter tended to send these animals deeper into the filter beds. The cycle of their appearance indicated that they were responsible for sloughing. As the algae returned after 3-4 weeks the worms moved upward to feed upon this growth. The more rapid regeneration

of the algae in the summer was also attributed to the effects of the worms. The value of the worms in developing sloughing conditions and in controlling the film was demonstrated experimentally. In the filters under study the worms were considered as important as the insect larvae, Psychoda severini, P. alternata, Metriocnemeus longitarsus, M. hirticollis, and Spaniotoma minima, in controlling accumulative film growth. The snail, Limnea glabra, as well as mites and spiders, also occurred on these filters.

Wilson (1943, 1949) reported that scouring organisms such as Macrobiotus sp., oligochaete worms, and insect larvae were important in preventing excessive development of film and increasing plant efficiency in Wisconsin and Minnesota. The worms listed included: Aelosoma hemprichi, Pristina sp., and Dero sp. He stated that while worms such as Dero were few in number they exerted a potent influence on the reduction and stabilization of sewage solids and assisted in clearing voids between rocks to the improvement of ventilation.

Inhoff and Fair (1956) indicated that among the worms present in trickling filters were the aquatic "earthworm" Limnodrilus sp. and the reddish bristle or sludge worm, Tubifex sp.

Usinger and Kellen (1955) studied the role of insects in several types of sewage disposal beds in California. In an experimental biofiltration plant at San Rafael Meadows insect larvae were found. The commonest species was a member of the Diptera, Psychoda alternata. Other Diptera included: Limonia simulans concinna, Diploneura cornuta, Scatella stagnalis, S. paludum, and Leptocera fontinalis. Cercyon fimbriatum, a member of the Coleoptera, was also found.

An experimental lath filter was set up in Iowa for the treatment of milk wastes. Frye and Becker (1929) studied the fauna of the slimes which developed on the lath at 1-foot intervals in the 6-foot deep filter. The organisms were presented in a combined list with the protozoans qualified as "common" or "rare". The list included: Protozoa: Sarcodina: Common: Dimastigamoeba gruberi, Vahlkampfia linas, V. albida, Amoeba sp., A. radiosa, Euglypha alveolata, Chlamydomorphys sp., Actinophrys sol, Centropyxis aculeata, and Arcella vulgaris; Rare: Amoeba sp., A. proteus, A. verrucosa and A. villosa; Mastigophora: Common: Monas sp., Cercobodo ovatus, C. longicaudata, C. sp., Bodo caudatus, B. lens, Distigma proteus, Peranema trichophora, and Trepmonas agilis; Rare: Mastigamoeba sp., Oicomonas socialis, Bodo mutabilis, Euglena sp., Anthophysa vegetans, and Tetramitus sp.; Infusoria: Common: Chilodon cucullus, Colpoda sp., C. inflata, Cinetochilum margaritaceum, Linotus sp., L. fascicola, Euplotes charon, Cyclidium glaucoma, Pleurotricha lanceolata, Oxytricha pellion ella, Uronema marina, U. sp., Vorticella sp., and Pleuronema chrysalis; Rare: Colpidium striatum, Glaucoma scintillans, Prorodon teres, Stylonychia pustulata, Podophrya sp., Paramoecium caudatum, and Opercularia berberina; Metazoa: Turbellaria (flat worms): Stenostomum leucopus; Rotatoria: Euchlanis dilatata, Philodina roseola; and Rotifer vulgaris; Gastrotricha; Chaetonotus sp.; Nematoda: unidentified; Annelida: Aelosoma hemprichi.

In certain areas, populations of snails can develop in trickling filters. Ingram et al. have reported (1958) that at least five species of snails have been found in the United States on trickling filters.

Physa anatina was found at Urbana-Champaign, Illinois, Physa halei at Fort Worth, Texas, P. cubensis at Gainesville, Florida, and P. integra at Dayton and Hillsboro, Ohio. In addition, Lymnaea humilis modicella was found once in low numbers at Urbana-Champaign. Serious damage to equipment was reported as a result of the activities of P. integra at Dayton and of P. cubensis at Gainesville.

It is not to be assumed that all organisms presented in the accompanying composite list (Table 46) are present on all trickling filters at the same time. Many of them occur in very low numbers indicating that they are transitory rather than members of the population at the time of sampling. Others are very abundant. When Hesseltine (1953) studied the trickling filters at Pearl River, New York, he found that Fusarium aquaeductuum, Geotrichum candidum, and Subbaromyces splendens were common enough to be considered members of the population, and that other species of molds which were present were considered chance contaminants. Becker and Shaw (1955) scraped surface stone of trickling filters at Pullman, Washington, and Moscow, Idaho, and found so few fungi that they decided that fungi were not part of the permanent population although Fusarium aquaeductuum was present at times in large enough amounts to produce orange colored patches on the filters. Fungi isolated from filter effluents indicated a certain amount of activity on the filters but the extent of this activity can only be guessed on the basis of work done on other filter populations.

Rate of Population Development

Before a complete description of the biota of the trickling filter can be given, information should be available on the development of the

Table 46. Composite list of trickling filter organisms

(Genera in major groups listed alphabetically regardless of importance or systematics.)

Schizomycetes (bacteria)

Sphaerotilus natans

Zoogloea ramigera

Many unidentified species

Cyanophyceae (blue-green algae)

Amphithrix janthina

Anacystis montana

Oscillatoria limosa

Phormidium uncinatum

Pleurocapsa sp.

Eumycophyta (Fungi)

Absidia corymbifera

cylindrospora

Allescheria boydii

Alternaria tenuis

Ascodesmia sphaerospora

Aspergillus spp.

candidus

clavatus

flavipes

flavus

fumigatus

niger

ochraceus

oryzii

terreus

ustus

versicolor

wentii

Cephalosporium spp.

Chaetomium globosum

Cladosporium cladosporioides

Coniothyrium spp.

fuckelii

Dematiaceae spp.

Epicoccum purpureogenum

Fusarium spp.

aquaeductuum

oxysporum

roseum

solani

Geotrichum candidum

Gliocladium roseum

Gliomastix convoluta

Leptomitus lacteus

Memmoniella echinata

Monilia sitophila

Moniliaceae spp.

Mucor spp.

fragilis

plumbeus

racemosus

saturninus

Myrothecium verrucaria

Paecilomyces varioti

Penicillium spp.

brevicompactum

chrysogenum

clavigerum

Penicillium cyclopium

digitatum

expansum

funiculosum

herquei

implicatum

janthinellum

lilacinum

?luteum

martensii

nigricans

ochrochloron

oxalicum

palitans

piscarium

purpureogenum

roquefortii

stipitatum

variabile

velutinum

vermiculatum

Phialophora jeanselmei

Phoma spp.

Aureobasidium pullulans

Rhizopus spp.

arrhizus

nigricans

rhizopodiformis

Rhodotorula spp.

Sphaeronema spinella

Sporotrichum pruinosum

Stachybotrys atra

Stemphylium consortiale

Subbaromyces splendens

Syncephalastium racemosum

Trichoderma viride

Trichothecium roseum

Verticillium spp.

lateritium

White yeasts

Chrysophyceae (Diatoms)

Nitzschia palea

Chlorophyceae (Green Algae)

Aeronema polymorphum

Characium sp.

Chlorella vulgaris

Chlorococcum humicola

Microthamnium kuetzingianum

Richterella sp.

Stigeoclonium nanum

Ulothrix tenuissima

Bryophyta - Musci (Mosses)

Leptodictyum riparium

Protozoa

Sarcodina (Amoeboid organisms)

Actinophrys sol
Actinosphaerium eichhornii
Amoeba proteus
 radiosa
 verrucosa
 villosa
Arcella dentata
 vulgaris
Centropyge aculeata
Chlamydomonas stercoraria
Cochlicopodium bilimbosa
Diffugia pyriformis
Distigamoeba gruberi
Euglypha alveolata
Hartmannella hyalina
Pomphagus nutabilis
Protamoeba primitiva
Trinema lineare
Wahlkampffia albida
 guttula
 limax

Mastigophora (flagellates)

Anthophrysa vegetans
Bodo caudatus
 lens
 mutabilis
Cercobodo sp.
 longicaudata
 ovatis
Distigma proteus
Euglena sp.
Heteronema sp.
Mastigamoeba sp.
Monas sp.
Notosolenus sp.
Oicomonas socialis
Peranema trichophora
Tetramitus sp.
Trepomonas agilis
 "Many small flagellates"

Infusoria (Ciliates)

Amphileptus sp.
Amphisia sp.
Aspidisca sp.
Blepharisma sp.
Carchesium sp.
Chilodon sp.
 cucullus
Cinecocchilum margaritaceum
Colpidium sp.
 striatum
Colpoda sp.
 inflata
Cyclidium sp.
 glaucoma
Euplotes sp.
 sharon
Glaucoma sp.
 scintillans

Halteria sp.
Holophrya sp.
Lembus sp.
Lionotopsis sp.
Lionotus sp.
 fascicola
Loxodes sp.
Loxophyllum sp.
Macrystaria sp.
Metopus sp.
Microthorax sp.
Nassula sp.
Opercularia sp.
Oxytricha sp.
 pallionella
Paramoecium sp.
 caudatum
Pleuronema sp.
 chrysalis
Pleurotricha lanceolata
Podophrya sp.
Prorodon sp.
 teres
Spirostonum sp.
Stentor sp.
Stylonichia sp.
 pustulata
Uroleptus sp.
Uronema sp.
 marina
Vorticella sp.

Metazoa

Turbellaria (Flat Worms)
 Stenostomum leucopus
Rotatoria (Rotifers)
 Euchlanis dilatata
 Philodina roseola
 Rotifer vulgaris
Nematoda (Nematode worms)
 Diplogaster strictus
 Diploscapter coronata
 Dorylaimus saprophilus
 Rhabdites sp.
Gastrotricha (Gastrotrich Worms)
 Chaetonotus sp.
Oligochaeta (Round Worms)
 Aeolosoma hemprichi
 Dero limosa
 Limnodrilus sp.
 Lumbricillus lineatus
 Lumbricus rubellus
 Pristina sp.
 longisetia
 Tubifex sp.
Polychaeta (Polychaete Worms)
 Polychaeta sp.
Crustacea
 Cyclops sp.
Mollusca

Table 46. p. 3.

Mollusca

Lymnaea humilis modicella

Physa anatina

cubensis

halei

integra

Tartigrada

Macrobiotus sp.

Arachnida

Torrenticula anomala

Insecta

Cercyon fimbriatus

Diploneura cornuta

Leptocera fontinalis

Limonia simulans concinna

Psychoda alternata

Scatella paludum

stagnalis

population. A method of sampling the algal and fungal components of the population during its developmental and mature stages has been described (Cooke, 1958; Cooke and Hirsch, 1958). Briefly the technique includes exposure of weighed glass slides on the filter stone, removal of the slides at weekly intervals, and study of the population developed there. The study is accomplished quantitatively by weighing air-dried material on the slides, by reviving some members of the population, plating them and counting the colonies or by directly counting cells and cell clusters in the materials which have been resuspended in water.

At the Dayton, Ohio, sewage treatment plant, studies were made simultaneously on a low-rate and a high-rate trickling filter. Sixty sets of frames, each with four attached slides, were placed on each filter. Slides were returned to the laboratory at weekly intervals for 57 weeks. Colony counts of fungi found in the rewet material were obtained for each slide from each filter. These are presented in Table 47 for the first 25 weeks for both filters. A mathematical curve, the logistic, was fitted to the population growth data, that is, colony counts from the start of observation to the time at which these counts appeared to level off. The equation for this curve is:

$$\text{Expected } y_t = \frac{k}{\frac{\alpha + \beta \log t}{1 + e}} = k \frac{e^{\alpha + \beta \log t}}{\alpha + \beta \log t + 1}$$

where y_t is the colony count at time t , measured in weeks, k is the plateau level, determined as the average of the first several colony counts (7 for the low-rate, 4 for the high-rate) after what appeared to be the leveling off time (15 weeks for the low-rate, 19 for the high-rate filter), and α

Table 47. Fungal species and colonies on exposed slides (first 25 weeks)

Date of Harvest	Week	Number of days Exposure	Low rate filter		High Rate Filter		Air Dry Weight (gm)	Number of Colonies	Air Dry Weight (gm)
			Number of Species	Colonies	Number of Species	Colonies			
11-29	1	7			10	1,662	0.33		
12-6	2	14	12	42	19	989	0.41		
12-13	3	21	12	41	19	740	0.74		
12-20	4	28	9	3,190	17				
12-27	5	35	12	1,845	21	47,002	0.96		
1-3	6	42	17	35,525	25	48,657	1.40		
1-10	6	49	23	40,463	23	56,694	1.43		
1-17	8	56	19	79,365	18	70,100	1.65		
1-24	9	63	16	124,157	16	174,125	1.35		
1-31	10	70	15	7,696,770	17	202,025	1.42		
2-7	11	77	18	451,675	23	168,175	1.61		
2-14	12	84	22	369,475	26	320,250	0.53		
2-21	13	91	20	564,130	25	344,800	0.69		
2-28	14	98	15	1,998,130	19	321,055	0.93		
3-7	15	105	16	2,030,975	19	1,473,675	0.64		
3-14	16	112	18	16,439,000	14	832,975	1.43		
3-21	17	119	14	9,929,750	16	3,136,750	1.51		
3-28	18	126	14	2,731,250	15	1,112,400	0.71		
4-4	19	133	12	9,077,050	15	1,654,750	1.05		
4-11	20	140	14	13,872,000	18	6,814,750	1.40		
4-18	21	147	12	7,161,750	12	14,131,250	1.82		
4-25	22	154	13	5,114,750	16	10,921,000	1.75		
5-2	23	161	12	10,610,000	15	13,413,250	2.20		
5-9	24	168	13	2,730,000	15	7,200,000	1.76		
5-16	25	175	12	13,495,000	17	5,575,000	1.78		
						7,935,000	1.50		

1954

and β are parameters (Fig.13). For the high-rate filter, k was found to be 12,800,000, and for the low-rate filter, k was 9,200,000.

Estimates of the parameters α and β are obtained by putting the above equation in the following linear form in terms of common logarithms:

$$\text{Observed } \log_{10} \frac{y_t}{k - y_t} = .43429 (\alpha + \beta \log t).$$

To ease the calculations, the factors $y_t/k - y_t$ were multiplied by 100,000 in the case of the high-rate filter and by one million for the low-rate filter.

Thus, if $\frac{y_t}{k - y_t} : = 100,000 \frac{y_t}{k - y_t}$ we obtain

$$\log_{10} \frac{y_t}{k - y_t} : = 5 + .43429 \alpha + .43429 \beta \log t$$

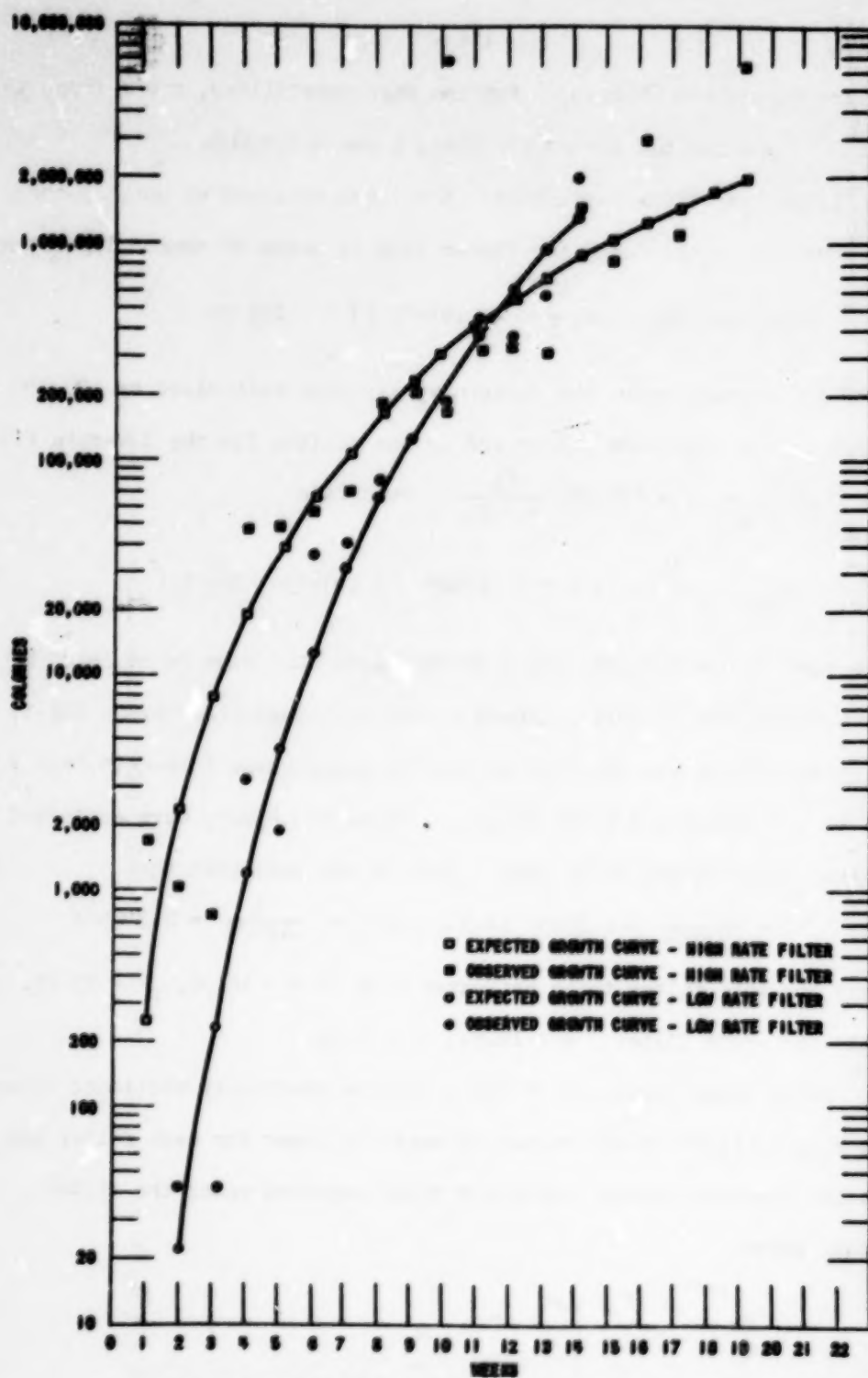
(In the case of one million, the 5 on the right hand side is replaced by 6).

Thus, plotting the observed numbers on the left-hand-side versus $\log t$, a good straight line was obtained on log-log paper whose intercept, say A , equal to $5 + .43429 \alpha$ and whose slope, B , equal to $.43429 \beta$, were estimated by the usual least square techniques. Then it was estimated that

$$\alpha = \frac{A - 5}{.43429} = 2.30259 (A - 5), \text{ and } \beta = \frac{B}{.43429} = 2.30259 B$$

For the low-rate filter these estimates were $\alpha = -16.92$, $\beta = 13.25$, and for the high-rate filter $\alpha = -10.87$, $\beta = 7.19$.

Using these values of α and β and the previously mentioned values of k , Figure 13 plots these values on semi-log paper for each filter and shows the observed colony counts and those expected under the fitted logistic curve.



COLONY COUNTS OBSERVED AND EXPECTED UNDER FITTED LOGISTIC CURVE

FIGURE 13

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It will be noted that the population growth curve rises more sharply and steeply on the low-rate than on the high-rate filter and would arrive at the upper asymptote, k , more quickly. In view of the fluctuations observed during later weeks, it is believed that there is, in the long run, no real difference between the two selected values of k . The logistic appears to be an adequate representation of the increase in the number of colonies during the build-up phase.

Description of the Biota

The community of organisms occupying a trickling filter, and the changes through which it goes as it develops from its inception or as a result of changes in dosing or in quality of applied sewage, can be described in terms of the formula given by Major (1951). His basic formula can be modified for application to this type of population as follows:

$$b = f_b (cl, p, r, o, t)$$

where b , the biota of a specific trickling filter, is a function of cl , the regional climate, p , the physical and chemical quality of the applied sewage at time zero, r , the fixed operational conditions of the specific filter, o , the biotic effect of the organisms of the community and of man, and t , time.

At any moment in time the biota of the filter becomes adapted to conditions of growth on the filter through natural selection rather than deliberate seeding of special segments of the population. The most important parts of this equation are thus p and o . The kind of sewage p applied to a filter will determine the members of the population, since

some species will not be able to tolerate some of the sewage materials because of toxicity or inability to use them as food. The factor of man's interference o_h with the population is important since dosing rates may vary, different types of sewage may be applied at different times, and other operational problems may interfere with one or another type of population. Factors of regional climate cl will be relatively constant within one filter although they may vary between different parts of the country or between different types of filter. Within one filter, or one filter field, the regional climate will not vary and will not be particularly important except to the immediate surface layer. Within one filter, temperature of applied sewage will be relatively constant and will be affected only slightly by air temperature. Precipitation in the form of applied sewage will be relatively constant except during periods of heavy rainfall when some of this water is diverted to the filters and is received in the form of more dilute sewage applied more rapidly than usual. Effects of such dilution are usually of brief duration and not of catastrophic importance. Some sloughing may result but recovery is relatively rapid. Effects of the r type would be noted on the filter by the differing populations on the surface layer of stone, because of the presence of light-sensitive algae and fungi, as contrasted with those populations at lower levels. Types of filter construction, ability to prevent ponding, and rate of application of sewage so far as diameter of distributor arms and stationary nozzles are concerned, would be noted in variations of r-type effects. Effects

of time t would vary only slightly if the sewage dosage throughout the year were relatively constant in composition and rate of application, and if the sewage were applied to a well designed and constructed filter. That is, throughout the year, one could expect a certain type of biota to develop on a trickling filter under relatively constant conditions of regional climate, including normal seasonal fluctuations, relatively uniform municipal or industrial waste applications, relatively uniform and continuous operation of the filter, and relatively uniform development of the population, including growth, death, and replacement of component parts, without undue interference from man.

Variations in populations occur when dosing rates are changed, type of sewage applied is altered chemically or physically, filters are stopped for servicing, flooding conditions are induced by excessive precipitation or in attempted control of insect or snail populations, or when other factors are altered. Changes in population patterns can be noted by casual macroscopic observation when excessive sloughing occurs, but such observations are not useful unless accompanied by detailed analyses of all aspects of sewage influents and effluents and of engineering factors involved.

Activities of Segments of Filter Bed Populations

It has been suggested, in connection with the listing of the several groups of organisms, that during the course of their life on or in the microbial film these organisms contribute to sewage purification. Upon and within each filter bed a balanced population develops. The population of the film is built up gradually, although the time required may be as short as 50 days (Gilcreas and Sanderson, 1940). Its members

are derived from various sources and received by the filter either from influent sewage, by initial seeding by the use of effluents from other trickling filters, or by settling of disseminules from the air upon the filter. Such members of the raw sewage population as are readily adapted to life on the filter settle out and become attached to the filter stone. If they can successfully compete with the population already present they will become members of the population of the microbial film.

The algae derive some mineral and nitrogen nutrition from the sewage and are close to an ample supply of carbon dioxide. In turn, they add oxygen to the water in which they are continually bathed. Bacteria and fungi are able to remove quantities of solid, colloidal, and dissolved organic materials from the water through metabolism and supplying a film onto which many of these materials can be adsorbed and thus effectively removed as the sewage passes through the filter. Protozoans and smaller metazoans easily devour large numbers of bacteria, thus removing members of that population and making space in that particular ecological niche for additional bacteria. This activity tends to control bacterial multiplication keeping it in a continual logarithmic growth phase. If the organism has a large enough "mouth", yeast cells may also be ingested and a similar control is effected on the yeast population. The larger metazoa, such as annelid worms, insect larvae, and snails, browse upon the larger fungal and algal populations. Although they are not necessarily carnivorous, the metazoa may ingest, at the same time, any of the smaller organisms and sludge materials adsorbed upon the biological film on which the larger metazoans are feeding.

The concluding statement by Ingram et al (1958) may be elaborated

as follows: In removing organic slimes through feeding, snails and annelids remove some percentage of B. O. D. as do various insect larvae. They can thus be looked upon as "crawling B.O.D. removed", as Psychoda and other flies and insects may be visualized as representing "flying B. O. D. removed". While the efficiency of this removal has never been evaluated satisfactorily, it is indicated by the large numbers of filter flies around trickling filter installations, and by the snails, as many as a ton, that can be removed from a trickling filter plant in a day. In terms of food chains, annelid worms, insect larvae, and snails represent the terminus of production on the trickling filter.

Using chemical and biological analyses of materials on a new trickling filter in New York State, Gilcreas and Sanderson (1940) were able to determine that there was a gradual increase in the degree of oxidation of organic and nitrogenous matter in settled domestic sewage at the temperatures received coincident with the gradual development of microbial populations. As a result of a study conducted during the first 107 days of operation it was found that complete oxidation of nitrogenous compounds was effected after 40 days, 50 days were required for total development of the characteristic biota of trickling filters, and 60 days were required for maximum reduction of carbonaceous organic matter.

C. E. Renn (1956) stated: "I am inclined to prefer Dr. Phelps' view of the role of the trickling filter to the more conventional conception of high oxidation rates on surfaces. We have no comparable experiences with other types of surfaces that justifies the extraordinarily

high rates of oxidation that are implied by assuming that the B. O. D. of a waste is removed in the interval of liquid passage. Rather, there is a great deal of evidence to show that we accumulate a large stock of organic material locally, through high rates of adsorption, and that the accumulated organic materials are oxidized at rates comparable to those of other organic systems."

Activities of the Microbial Film

A trickling filter is so designed that a large amount of biological film is exposed to the flow of sewage. Depending on plant design, this liquid material has passed through preaeration and settling tanks, as at Dayton, so that a portion of the solid, colloidal, and dissolved organic materials has been acted upon and settled out before the liquid reached the filters. However, a considerable amount of dissolved, colloidal, and solid material remains. As the liquid passes over or through the film, some of these materials are removed through metabolic processes of microorganisms and sometimes of macroorganisms, and additional portions are removed by adsorption on the film. The organic nitrogen content is a measure of the decomposable materials present at any time or place and is used together with B. O. D. as an indicator of the purification effected within a process.

Is it possible to relate the extent to which the removal of organic matter and the mineralization of various constituents of sewage materials is effected by the organisms of the microbial film to the numbers and types of organisms on the surfaces of the filters at the Dayton treatment plant?

Some idea of the potentialities of this question may be gained from the fact that a single trickling filter, 165 feet in diameter, 7.5 feet deep, is filled with 160,270 cu ft of limestone slag rock of varying sizes, usually 3-5 inches in diameter, length, or width. A flat circular surface 165 ft. in diameter comprises an area of 20 million sq. cm. The upper surface of the stone on a trickling filter is even more extensive because of the irregularities of the stone. The sampling technique includes 75 sq. cm. of glass slides on which determinations were made at weekly intervals. Such sampling took into consideration only the initial contact of the sewage with the microorganisms and did not consider the subsequent flow of the sewage through a 7.5 foot column of rock. Levine (1938) has shown that as sewage passes downward through the filter the nature of the materials in it becomes progressively different resulting in gradual downward change in the biota acting upon it.

Preaeration tanks were placed in service at the plant in July, 1954, but the complete effect of this type of early stage treatment, preceding primary settling, was not felt on the filters until the following May. This delay may have resulted from the further complication that the filter beds were temporarily by-passed, because of repair shutdowns for three periods between January and April. Throughout the study periods, the high-rate filters took a maximum load of sewage while the low-rate filters received only the excess. Materials entering any of either type of filter were not metered, nor is there a record of the hours or of days during which low-rate filters were not operating. Low-load periods occurred on weekends and during the night. Since effluents

from the two types of filters were not kept separate, data for filtered sewage represent a composite from both types. On the other hand, only data from the slides exposed to the surface of a high-rate filter are used in comparing results obtained on analysis of filtered sewage composited from both filters. On the basis of such data, and because only the surface of the beds was sampled, it may be surprising to find any correlation between air-dry weight of organisms and amount of activity produced by such organisms.

Using available climatic and plant records (Fig.14), an attempt was made to determine whether the average weekly air temperature (Vandalia, Ohio), the average monthly sewage temperature, and the total weekly precipitation (sum of the higher of the two daily recordings from Vandalia and the sewage treatment plant) showed correlation with the amount of growth on the slides as indicated by average weights of material on four slides from each of the high- and low-rate filters.

Weight per slide. - Certain discontinuities in the growth curve were traced to periods when the plant was shut down, or the filters by-passed. Enough time was subtracted from those periods associated with readings following shut-downs to make the weight on the slides immediately following the shut-down coincide with the weight of the original build-up preceding the shut-down. It was found that growth following a shut-down was noticeably slower than that preceding a shut-down when the same weight of material occurred on the slides (Fig.15). Straight line curves fitted to the data on log-log paper

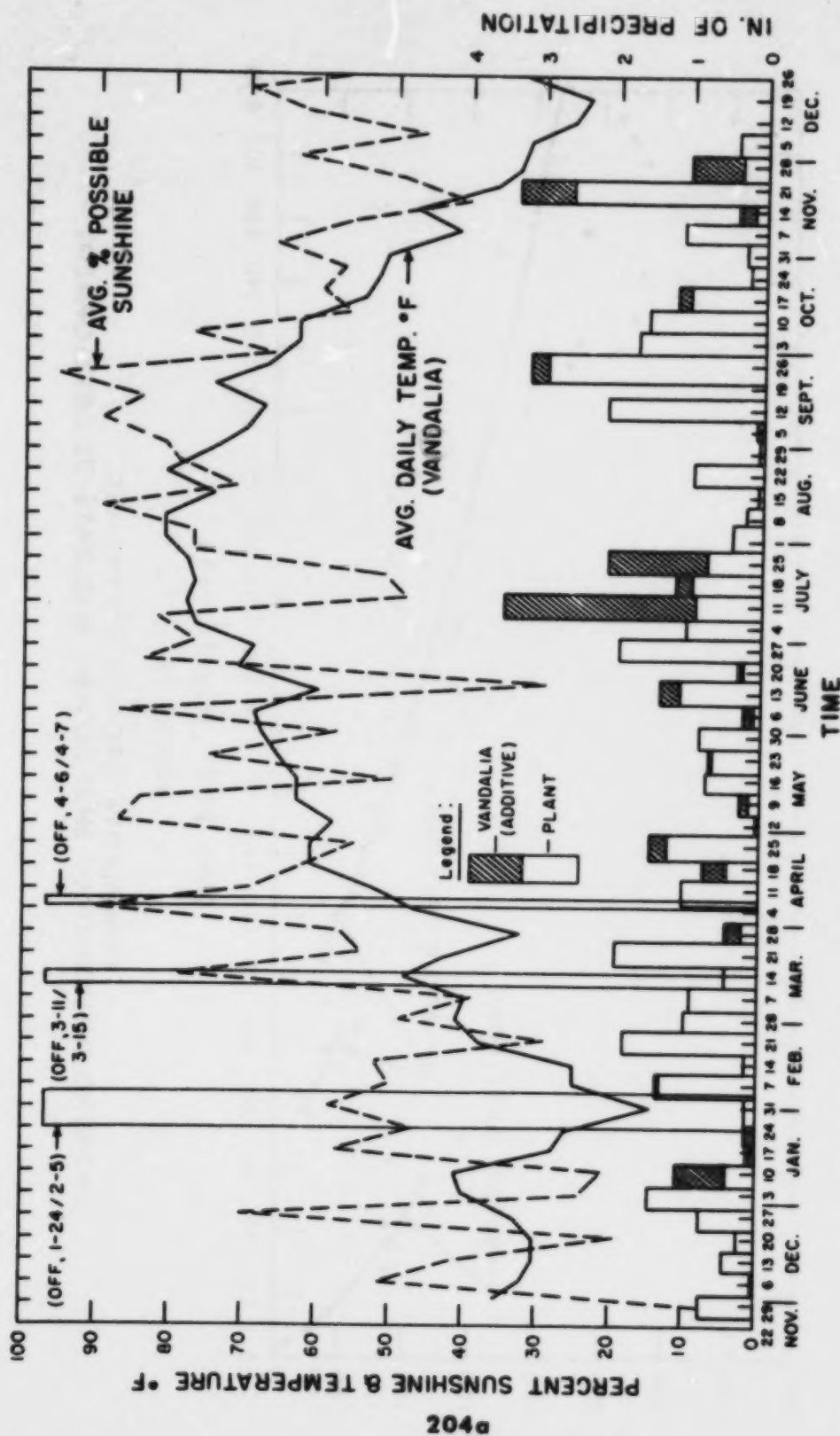
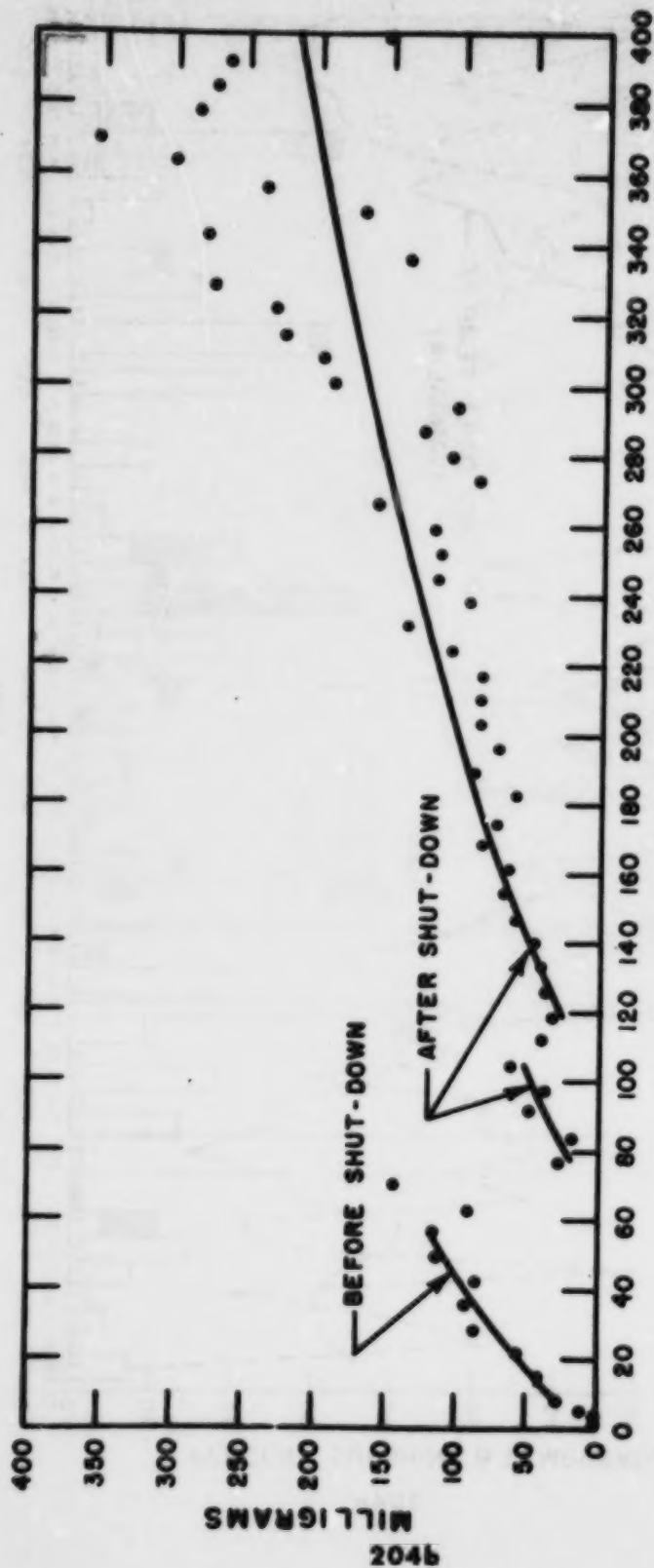


FIGURE 14 - WEEKLY AVERAGES OF AVERAGE DAILY TEMPERATURE, AVERAGE PERCENTAGE POSSIBLE SUNSHINE, AND TOTAL EFFECTIVE PRECIPITATION FOR THE DAYTON AREA.



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FIGURE 15

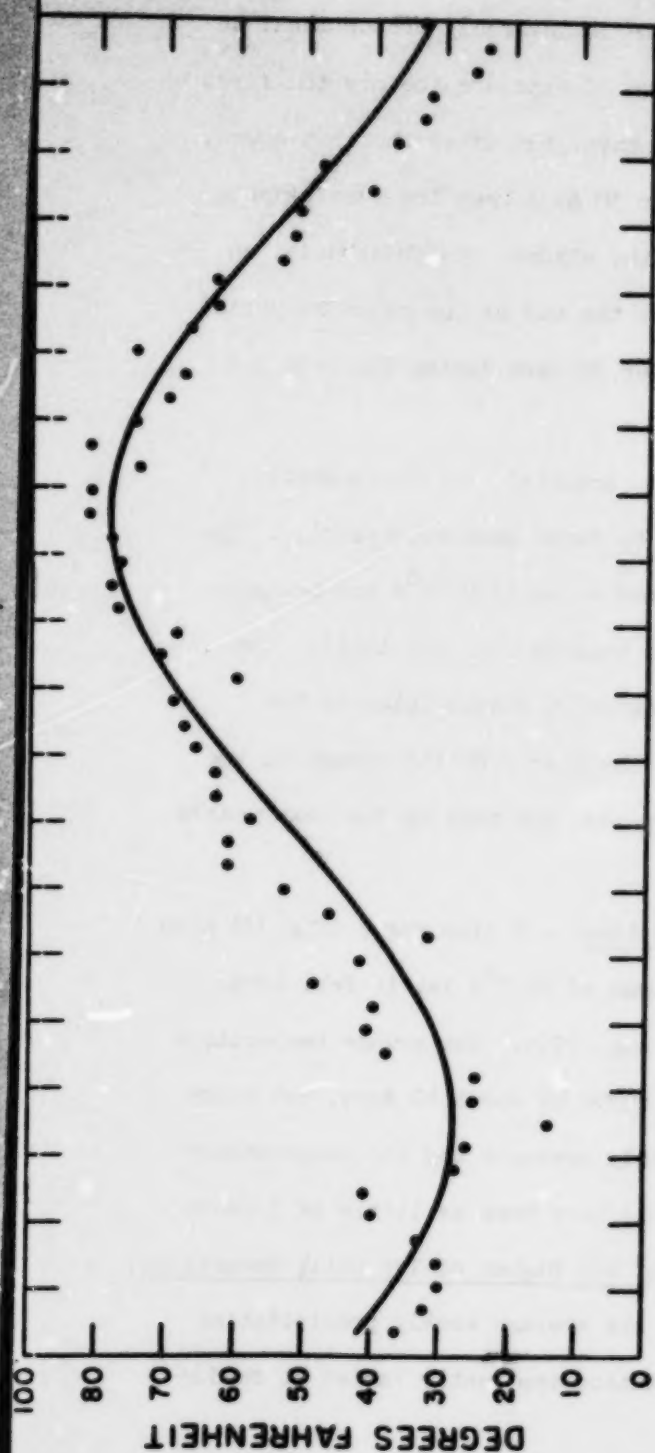
CUMULATIVE GROWTH ON FRAMES
SHOWING INFLUENCE OF SHUT-DOWNS AND RATE OF DEVELOPMENT.

show that the percentage growth rate is inversely proportional to time. Growth during the first month of exposure (before the first shut-down) was about 61 mg. per 30 days, but after the shut-downs, the growth rate was only 35 mg. per 30 days when the same weight, (25 mg.) of organisms occurred on the slides. On this basis, an equilibrium had not been reached at the end of the exposure period since the growth rate was 17 mg. per 30 days during the 12th and 13th month.

Average Weekly Air Temperature (at Vandalia). - A trigonometric sine function was fitted (Fig. 16) to these data successfully. The minimum (trough) value of the fitted curve is 28.2°F and occurred in the 10th week of the experiment (ending Jan. 31, 1955). The maximum (peak) value of 78.2°F occurred 6 months later in the 36th week (ending Aug. 1, 1955). The rise from the trough to the peak is a mirror image of the drop from the peak to the next year's trough.

Average Monthly Raw Sewage Temperature. - A sine curve (Fig. 16) also fitted these data well. The minimum of 59.7°F lay in Feb. 1955, and the maximum of 75.6°F lay in Aug. 1955. The sewage temperature curve lagged the air temperature curve by about 40 days, but since the sewage temperatures were monthly averages and air temperatures were weekly averages, the lag could have been as little as 3 weeks.

Total Weekly Precipitation (Sum of the Higher of Two Daily Recordings). - (Fig. 14). There was no trend in the average weekly precipitation during the year. Weekly precipitation apparently varies at random



205a

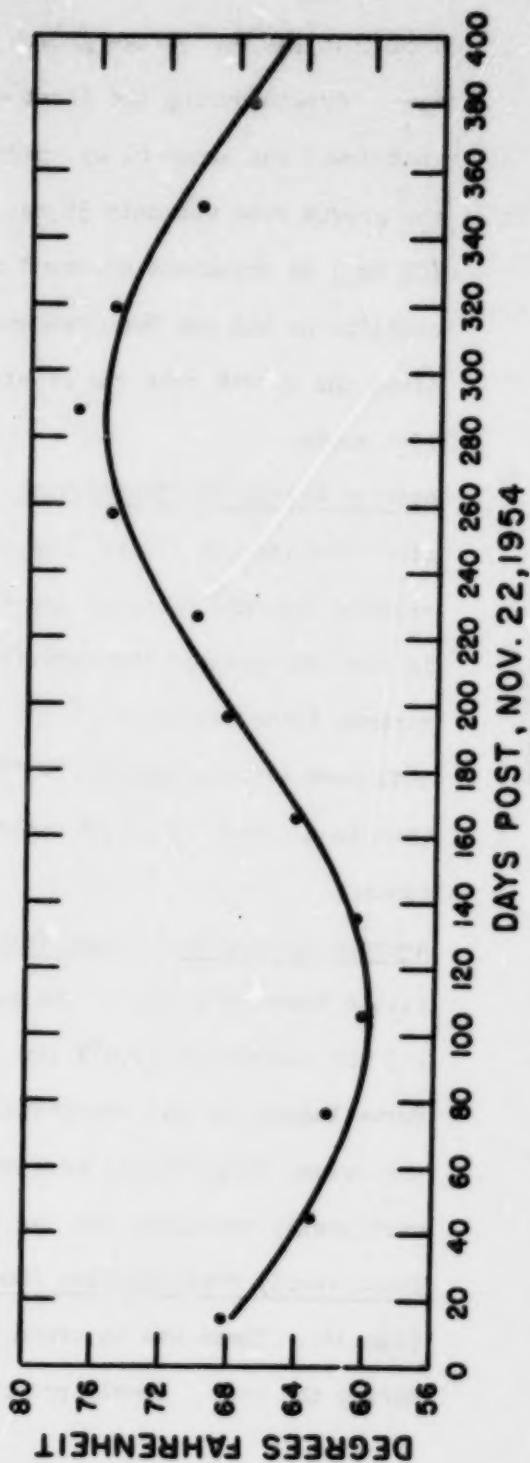


FIGURE 16
AVERAGE WEEKLY AIR TEMPERATURE (VANDALIA, OHIO) AND AVERAGE MONTHLY
TEMPERATURE OF RAW SEWAGE (DAYTON).
DAYS POST, NOV. 22, 1954

an average of 0.90 inches per week, but is not symmetrically distributed about this average. That is, there were more values below the average (35) than above (22).

Correlations. - Several statistical techniques were tried to determine whether or not the microbial film was affected by the climatic conditions to which it was subjected throughout the year. No evidence exists to indicate a relationship between the irregularities (sloughing or unusually rapid growth) in slide weights and corresponding deviations from seasonal patterns of the weekly average air temperature, raw sewage temperature, or total weekly precipitation figures.

Chemical and physical analyses of sewage. - A chemical and physical check is maintained by the chemical laboratory of the Dayton sewage treatment plant on the effectivity of the treatment. At four stages in the treatment process - raw, settled, filtered, and final - the B.O.D., dissolved nitrogen, ammonia nitrogen, organic nitrogen (Fig. 17, expressed as removal), chlorides, and suspended solids (Fig. 18) are analyzed. The 5-day B.O.D. and suspended solids are measured daily at each of these points. The other values are taken on the 1st, 8th, 15th, 22nd, and 29th of each month unless one of these dates happens to fall on or near a holiday. On the accompanying graphs, values for these factors are plotted as near the day on which sampling was done for the week as possible. Values used for the present study include only those for settled sewage entering

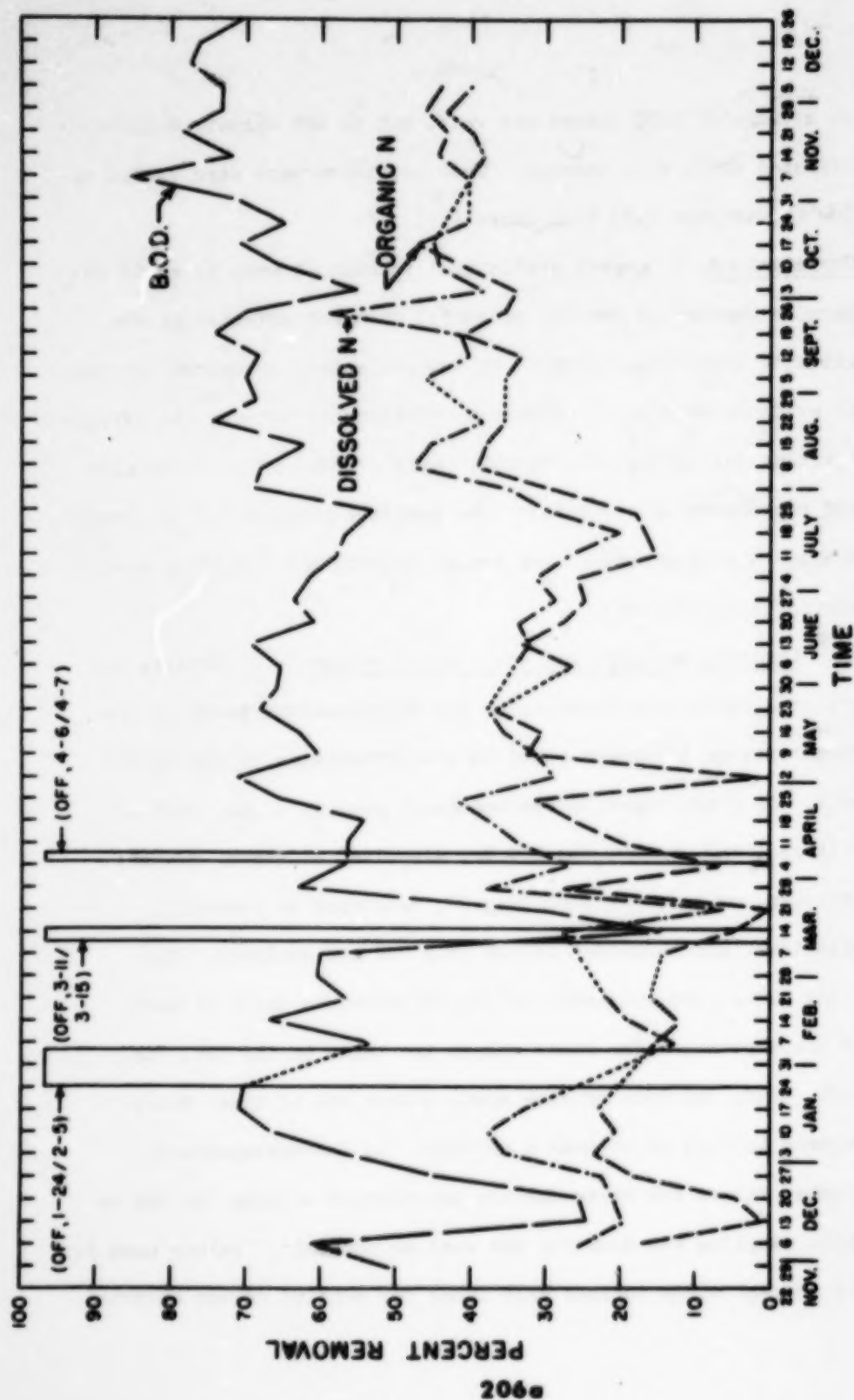


FIGURE 17 PERCENTAGE REMOVAL OF B.O.D., DISSOLVED NITROGEN AND ORGANIC NITROGEN DURING FILTRATION.

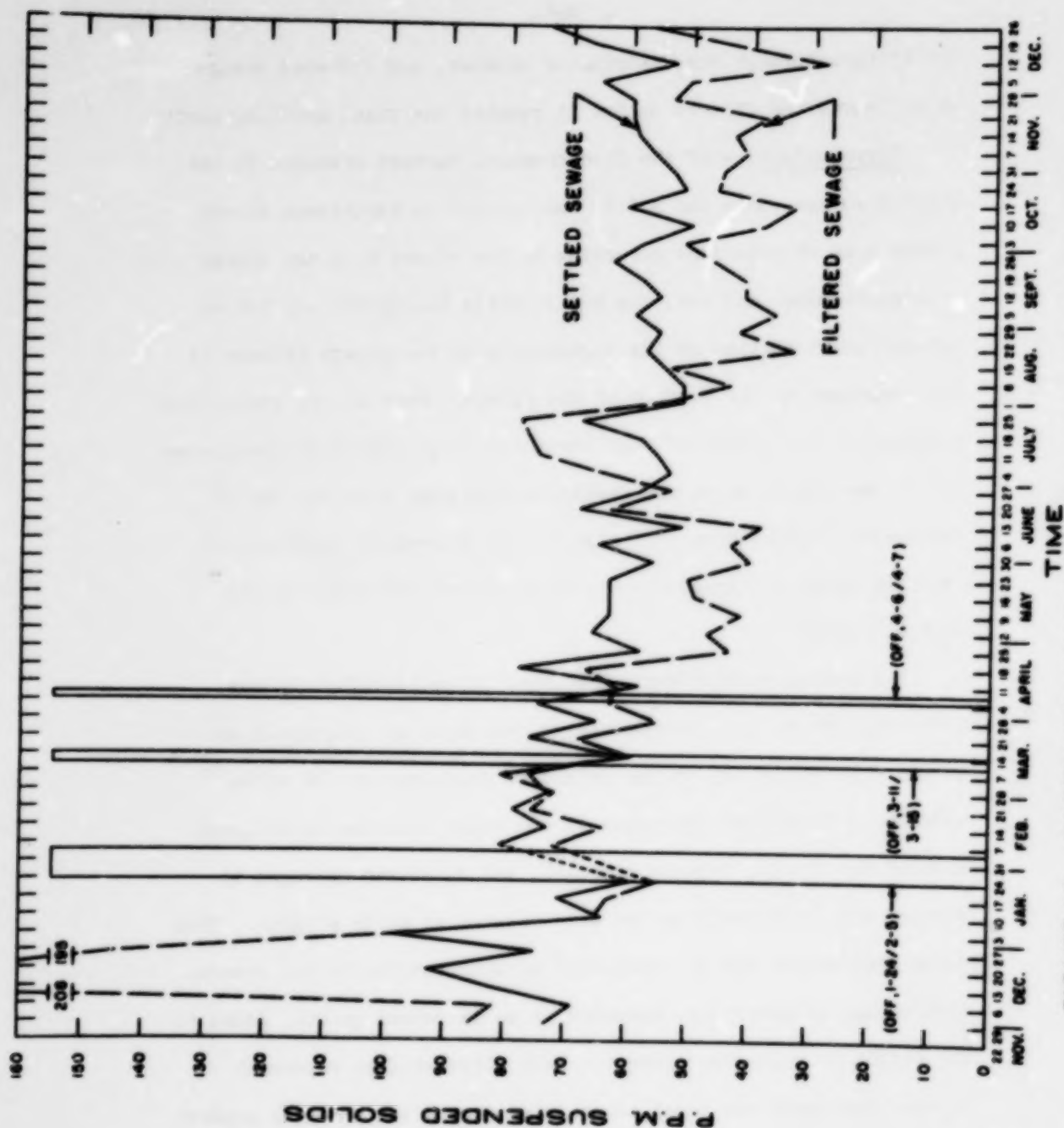


FIGURE 18 - SUSPENDED SOLIDS IN SETTLED AND FILTERED SEWAGE.

the filters through the distributor nozzles, and filtered sewage, as it leaves the filters before it reaches the final settling basin.

Correlations. - Of the five chemical factors measured in the settled sewage, only the B.O.D. was related to variations in the growth rate of organisms occurring in the slimes with any degree of significance, and even the B.O.D. fails to explain any but an insignificant portion of the variability in the growth pattern of the organisms on the surface of the filter. Most of the statistical analyses of the effect of the chemicals on growth of microorganisms and of the organisms on the chemicals were made with the use of regression coefficients. No significant regression coefficients of slide weights themselves were noted on the chemicals in the settled sewage.

Regression coefficients were found to be satisfactory for assessing the possible dependence of the chemical concentrations in the filtered sewage on the weight of organisms on the slide surfaces. Using this technique it was found that the differences between the B.O.D., organic nitrogen, and dissolved nitrogen in settled and in filtered sewage were related to slide weights. Thus these differences can be postulated as attributable to the removal from sewage of materials, adsorbed or as increased growth, added to the weight of organisms present. Such slide weights represent neither increment nor total accumulated growth but material present at any one date following addition by growth or adsorption, as well as loss by death and sloughing. On the other hand, the amounts of

ammonia nitrogen and suspended solids in either the settled or filtered sewage did not appear to be related in any way to the organisms on the surface of the filter. In the case of suspended solids, this observation is of interest since a good proportion of these solids is composed of sloughing from the microbial film.

Adaptation to Habitat

The organisms which live in the sewage treatment plant, especially those on the trickling filters, have become adapted to life in a waterfall-cascade system in which the effluent from primary settling tanks is the liquid passing over the rocks. This liquid contains a quantity of suspended, colloidal, and dissolved materials, primarily waste materials resulting from human metabolism but also including such wastes as laundry and wash water, industrial wastes, soil run-off, and wastes from garbage disposal units. Fundamentally, there is little difference between a habitat in which such materials are present and a soil habitat which contains fragments of dead plant or animal parts, as well as the excrement of various native animals. Many, if not all organisms which live on trickling filters have become adapted to this condition of existence following the development of a capacity to live all their lives in the presence of water.

According to various interpretations of the Kolkwitz and Marsson (1911, 1950) system of classification of zones of pollution in streams (Whipple, Fair and Whipple, 1947), the solids removed

from sewage during the settling process and treated in sludge digesters form a polysaprobic habitat while the settled liquid passing over the trickling filter provides a habitat interpreted as ψ-mesosaprobic. Thus all of the organisms found living in a trickling filter (Table 46) could be interpreted as ψ-mesosaprobic. However, there appear to be different degrees of adaptation to such an environment. Cooke (1957a, b) has indicated that for the fungi there may be four different types of response to the pollutional habitat. The terminology defined in these papers is not necessarily restricted to the fungi but may be applied to all organisms living in the habitat. Those species which have never been reported except from trickling filters, dung, decaying animal bodies under water, and similar habitats may be considered lymbionts. At least three species of fungi (Pilobolus sp., Ascodesmis sphaerosporum and Subbaromyces splendens), Tubifex worms, and possibly some bacteria and protozoans may be included in this category.

Most of the organisms found on trickling filters may be considered as lymphiles. They are closely associated with this type of habitat, although in certain instances they may be found in other habitats where decay occurs or where conditions are tolerable.

Of the fungi, at least, a large number of species, represented by a small number of individuals, are considered to be lymaxenes. These organisms are well adapted to living habitats other than those which may be considered to be sewage polluted, but

for short periods of time they can join the other members of the trickling filter population. At such times they may be passing through the plant as chance contaminants and become trapped in the slime by adsorption or by mechanical screening methods.

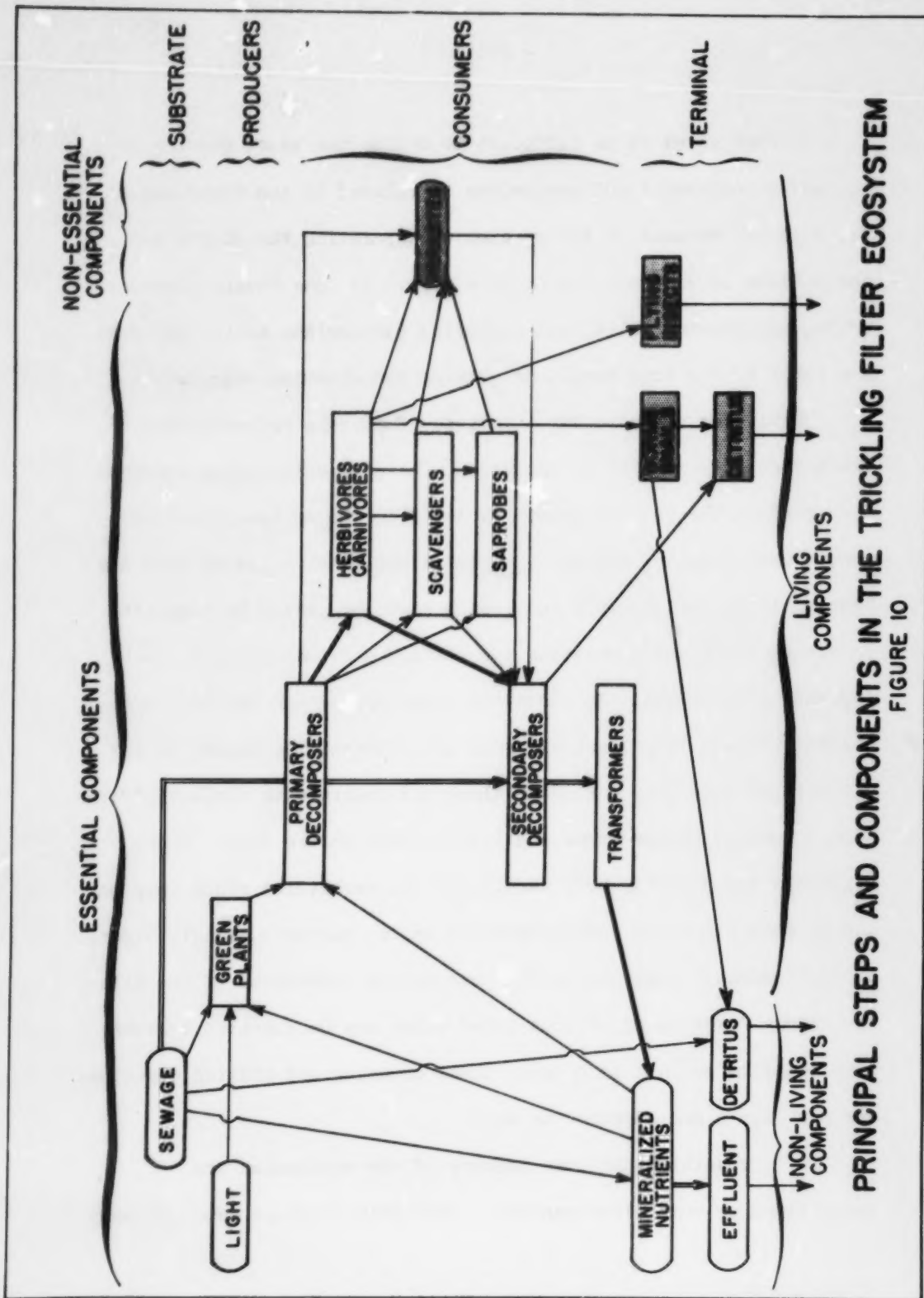
One should never find a lymaphobe in this habitat at least in vegetative condition. Such a find would be the result of a chance contamination, a spore being carried through the plant by the water in which it was accidentally picked up.

While much of the biology of algae, protozoans, metazoans, and bacteria which are found on the filters as members of the population is not well known, it may be assumed that these organisms, too, will be found to fall into the biological classes mentioned above. Trickling filters, as such, have been in existence for about 85 years (Stanbridge, 1954), and it does not seem likely that a whole new population of organisms could have developed during that time. It seems more probable that members of other populations have become adapted during this period of time to life in this specialized habitat. Prior to the formal recognition of the need for sewage purification in the late nineteenth century, sewage disposal or removal was a more or less individual matter, and where sewer lines existed they only carried the wastes to the nearest streams. Certain members of the population of organisms adapted to a sewage habitat may have originated from the biota of such waste-carrying channels.

The Ecosystem

The ecosystem of the trickling filters may be summarized by the chart presented in Fig. 19. This has been adapted from Clarke (1954) whose original chart has been modified to describe the specialized ecosystem under consideration. In this chart it is assumed that the trickling filter whose population is being described is not covered and that direct sunlight is being supplied to the algae which are the only green plants functioning in the treatment of sewage. It is indicated that the sewage being applied is an essential component since the population is developed for the treatment of this material. A somewhat artificial pair of categories has been introduced by the splitting of "decomposers" into primary decomposers, those which act directly on the sewage components, and secondary decomposers, those which act on dead tissue formed through development of the biological film.

From sewage, green plants can obtain certain required mineral supplies as well as nitrogen and phosphorus so that in the presence of light growth can be effected. It is possible that within the microenvironment there is present a larger amount of carbon dioxide than in many other habitats because of the great numbers of microorganisms existing in an actively metabolizing state. Those green algae which occur on the filter, then, are probably conditioned to a slightly higher percentage of atmospheric carbon dioxide than other algae or than algae of the same species in a different aquatic habitat.



PRINCIPAL STEPS AND COMPONENTS IN THE TRICKLING FILTER ECOSYSTEM

FIGURE 10

FIGURE 19 - PRINCIPAL STEPS AND COMPONENTS IN THE TRICKLING FILTER ECOSYSTEM.

In some cases it is difficult to define the exact meaning of the terms herbivores and carnivores as related to the organisms on the filters, because of the problem of separating the plants and the animals in a population of this type. In some cases, pigmented cells, unpigmented cells, and nonliving particulate matter all form the basis of the food supply of grazing and scouring organisms.

Herbivores and carnivores include protozoans and metazoans which feed only in part on bacteria. These organisms thus exercise a control on the rate of growth of the film and on the amount of growth which may be present. Colpidium and other ciliates keep the bacterial populations in a continuing state of growth by ingesting bacterial cells and permitting the continued rejuvenation of that segment of the biota. In addition, other protozoans may act in a similar way and larger animals such as rotifers, may ingest larger unicellular organisms including algae and yeasts with similar effects. Insect larvae, sludge worms and snails graze on the film. These organisms are probably both carnivores and herbivores since they may ingest both pigmented and unpigmented cells, bacteria, fungi, algae, and protozoans. Adsorbed on the less motile components of the film are sludge deposits of various types which may be ingested together with the film so that in a sense these browsing and grazing organisms are scavengers and saprobes as well.

It is possible that some members of the population are parasitized by some microorganisms. Such relationships have not been

shown directly, but in the examination of algal material submitted to him for identification, Drouet (in Cooke and Hirsch, 1958) indicated that Pleurocapsa-like material could have been a parasitized form of an Anacystis-like species. Characium sp. was found growing attached to Stigeoclonium nanum but this was probably an epiphytic relationship. While a number of types of bacteria and fungi are known to be able to attack members of the trickling filter biota in other habitats, parasitic relationships between the members of this biota and each other or additional invaders have not been observed. Rather, a mutualistic relationship has been established especially between the unicellular colonial organisms and the filamentous organisms in which the byproducts of the metabolism of one type of species are removed by another, thereby reducing potential toxic effects.

Scavengers and saprobes may degrade the dead tissues of the primary decomposers as well as the components of the sewage, but in turn they are subject to decomposition by the secondary decomposers whose byproducts are transformed into mineral nutrients which may also be added to the influent sewage to help restart the cycle.

Summary and Conclusions

The trickling filter is an ecological tool of the sanitary engineer in the treatment and purification of domestic sewage and industrial wastes and terminology developed in its use is described. The biota of trickling filter slime has received little attention as a working population unit although various segments of that unit have been described.

The populations of several trickling filters in the United States and England are reviewed according to limited studies based on either bacteria, fungi, algae, protozoans, worms, insects, or snails. An integrated list is presented to indicate something of the complexity of the biota.

A series of slide exposure samples of trickling filter biota has been studied with special reference to algae and fungi. As a result of fungal colony counts made from revived growths, a logistic curve has been developed to describe the rate of development of the population. The more rapid and steeper rise of the curve on a low-rate than on a high-rate filter is more apparent than real since the same curve is obtained in plotting the lower values of the two curves in parallel, so that no actual difference was noted between the two types of filter.

The functional, factorial description of a community can be used to describe the biota of a trickling filter. Climate is relatively uniform for a single filter except in periods of excessive precipitation or run-off. The biotic effects within the community are limited to replacement phenomena should an organism be unable to meet competition, and to the fact that grazing organisms tend to control the rate of population growth. Operational conditions of a specific filter, man's effect on a filter as operator and controller of the process, and the physico-chemical nature of the applied sewage would be effective in time, but at the end of a study of samples accumulative up to one year the effect of time itself had not been completely discovered.

Filter bed populations are effective in removing a large percentage of solid, colloidal, and dissolved matter in settled sewage, thus reducing the BOD of that sewage. This removal is not by metabolic processes alone but by adsorption on the film as well.

Growth on the stones of the filter develops rapidly at first but slows down as the film matures. In the case of reported experiments, points at which such slowing was most noticeable coincided with periods during which the filters were shut down for repair. No correlation between surface growth rate and total weekly precipitation, average weekly air temperature, and average monthly sewage temperature was noted. Surface growth was not correlated with differences between settled and filtered sewage of ammonia nitrogen, organic nitrogen, chlorides, or suspended solids. However, a slight correlation was noted in the case of BOD removal.

As a whole, the habitat is γ -mesosaprobic. However, different component organisms vary in their ability to tolerate the conditions to which they are exposed. Species represented most abundantly are lymaphiles. Many species, represented by few individuals, are lymaxenes, while the lymabionts have not yet been noted elsewhere. Lymaphobes are not expected in this habitat although an occasional spore or resting body may be observed.

The total ecosystem includes a few photosynthetic algae but mostly organisms dependent on preformed organic matter. Primary decomposers use such materials as food supplies, may be acted upon by herbivores or carnivores, or may be parasitized. At death all organisms of the system may be attacked by scavengers or saprobes which act as secondary decomposers whose products are transformed into mineralized nutrients which may reenter the metabolic system.

E. Other Trickling Filter Plants

Glendale. In addition to the preliminary sampling of slimes on the surface stone of the Glendale, Hamilton County, Ohio trickling filters, several sets of samples including primary settled sewage, slimes scraped from surface stones, and filtered sewage were obtained. On the basis of colonies observed in pour plates made from these samples, populations and amounts of fungal growth obtained were not significantly different from those observed at Dayton (Table 48).

Loveland. Two sets of samples were obtained from the Loveland, Hamilton-Clermont County, Ohio sewage treatment plant. At the time of sampling the plant served a community of fewer than 5,000 persons and several small industries including, especially, a slaughter house whose effluents occasionally upset activities in the sludge digester. The Robert A. Taft Sanitary Engineering Center used sewage at this plant in various types of experimental pilot-plant treatment systems. One of these was an anaerobic contact filter. A sand filtration process was also used

Table 48. Fungi observed in occasional samples from other trickling filter-type sewage treatment plants.

Species	Observation number*
White yeasts	46
<u>Geotrichum candidum</u>	37
<u>Phialophora jeanselmei</u>	25
<u>Fusarium aqueductum</u>	21
<u>Penicillium</u> spp.	19
<u>Cladosporium cladosporioides</u>	17
<u>Aureobasidium pullulans</u>	16
<u>Penicillium lilacinum</u>	16
<u>Rhodotorula</u> spp.	16
<u>Phoma</u> spp.	16
<u>Trichoderma viride</u>	14
<u>Alternaria tenuis</u>	12
<u>Mucor fragilis</u>	12
<u>Aspergillus flavus</u>	10
Moniliaceae	9
<u>Aspergillus niger</u>	8
<u>Aspergillus versicolor</u>	8
<u>Penicillium funiculosum</u>	6
Phycomycete	6
<u>Fusarium solani</u>	6
<u>Fusarium oxysporum</u>	5
<u>Mucor plumbeus</u>	5
<u>Paecilomyces varioti</u>	5
<u>Penicillium ochrochloron</u>	5
<u>Aspergillus ochraceus</u>	4
<u>Gliocladium roseum</u>	4
<u>Penicillium expansum</u>	4
<u>Arthrobotrys musciformis</u>	3
<u>Aspergillus flavipes</u>	3
<u>Epicoccum purpurascens</u>	3
<u>Aspergillus clavatus</u>	2
<u>Chaetomium globosum</u>	2
Dematiaceae	2
<u>Syncephalastrum racemosum</u>	2
<u>Trichosporon</u> sp.	2
<u>Verticillium</u> sp.	2
<u>Verticillium lateritium</u>	2

The following species were observed only once in this series:

Aspergillus candidus, A. fumigatus, A. sydowii, A. terreus, Gliocladium fimbriatum, Monosporium apiospermum, Mucor alternans, Myrothecium verrucaria, Penicillium janthinellum, P. oxalicum, P. stipitatum, P. variabile, P. velutinum, Phialophora sp., Preussia microspora, Rhizopus arrhizus, Saprolegnia sp., Sporotrichum sp., Stachybotrys atra, Stilbella sp., Mortierella hygrophila.

* Based on 32 samples from 17 habitats on 8 dates from plants at Loveland and Glendale, Ohio; Frankenmuth, Michigan, and experimental filters at the Taft Center.

on an experimental basis. Populations of fungi obtained at each visit were similar to those obtained from the Dayton plant at the comparable sampling points. Where points were not comparable, samples obtained yielded populations which varied only in kinds of fungi which yielded lower numbers of colonies. Species in the groups which were routinely present in relatively large numbers were similar in both plants and throughout the system. At one sampling occasion, insufficient settled sewage reached the trickling filter nozzles to form an effective filter-wide spray; instead, effluent from the nozzles poured directly down on the stones. When this type of operation occurred concentric ribbons of green algal growth were evident on the filters. Associated with the obvious green algae were the usual populations of bacteria, fungi, and protozoans.

F. Activated Sludge Plants

Because of distances involved, few activated sludge plants were studied for fungal populations. One of those closer to Cincinnati was at Yellow Springs, Greene County, Ohio, and it was sampled only five times. The largest one in the area is at Columbus, Franklin County, Ohio which was only sampled once. Later, but after other projects were using more time, a plant was placed in operation at Hamilton, Butler County, Ohio, and one set of samples for a study of yeast enumeration was obtained from it. No samples were obtained from the Wilmington plant.

Yellow Springs, Ohio. Five sets of samples were obtained from this small activated sludge plant which served a community which grew more rapidly than the capacity of the plant. Samples were obtained of raw sewage, primary settled sewage, sewage from the first and last aerator tank, scums which grew on the wall of the final settling tank, digesting sludge, air drying sludge, plant effluent, and water in the creek carrying the effluent to Glen Creek.

Samples were returned to Cincinnati and plated out on the day of collection. The same techniques were used in handling these samples as were used for those from the Dayton treatment plant. The same populations were obtained from the several types of samples as were obtained from the Dayton plant. The activated sludge yielded the same kinds of fungi, in relatively similar numbers, as the slimes on the trickling filters.

Columbus, Ohio. This plant was visited only once but a larger number of samples were taken than at Yellow Springs because of the larger size of the plant resulting from the need for more effective handling of larger amounts of sewage. Again, populations of fungi in the various processes sampled were little different from those which had been studied in other treatment plants. Unfortunately, at this stage in our studies, careful microscopic examination of the activated sludge floc was not a regular procedure. However, casual observation showed the presence of fungus filaments, an observation confirmed by the results on the pour plates.

Results of observations of fungi in sewage treatment plants are presented in Table 49, A and B.

Table 49a. Fungi observed in all types* Activated Sludge plants studied.

Species	Observation Number in 63 samples	Sample Dates (7)	Sample Areas (22)
<u>Geotrichum candidum</u>	98	7	18
<u>Moniliaceae</u>	78	6	17
<u>White Yeasts</u>	76	5	17
<u>Penicillium lilacinum</u>	67	7	21
<u>Aureobasidium pullulans</u>	58	7	18
<u>Penicillium spp.</u>	57	5	16
<u>Rhodotorula spp.</u>	50	6	17
<u>Fusarium aqueductum</u>	47	6	19
<u>Phialophora jeannei</u>	46	6	19
<u>Penicillium ochrochloron</u>	42	6	11
<u>Torulopsis candida</u>	31	1	7
<u>Trichoderma viride</u>	28	5	10
<u>Aspergillus versicolor</u>	26	5	11
<u>Cladosporium cladosporioides</u>	26	5	14
<u>Mucor fragilis</u>	23	6	12
<u>Penicillium funiculosum</u>	23	6	9
<u>Candida parapsilosis</u>	21	1	7
<u>Phoma spp.</u>	22	5	13
<u>Candida tropicalis</u>	19	1	4
<u>Candida krusei</u>	17	1	6
<u>Alternaria tenuis</u>	15	5	11
<u>Trichosporon spp.</u>	15	5	12
<u>Cephalosporium spp.</u>	16	4	7
<u>Aspergillus niger</u>	13	3	7
<u>Penicillium chrysogenum</u>	12	4	8
<u>Fusarium solani</u>	12	5	6
<u>Aspergillus fumigatus</u>	10	5	6
<u>Paecilomyces varioti</u>	10	5	9
<u>Trichosporon cutaneum</u>	10	1	5
<u>Fusarium oxysporum</u>	9	5	7
<u>Penicillium janthinellum</u>	8	4	4
<u>Aspergillus flavus</u>	7	4	7
<u>Sepedonium sp.</u>	7	3	5
<u>Torulopsis lanata</u>	7	1	4
<u>Torulopsis glabrata</u>	7	1	5
<u>Monosporium apiospermum</u>	7	3	5
<u>Aspergillus candidus</u>	6	1	6
<u>Mucor plumbeus</u>	6	4	4
<u>Penicillium cyclopium</u>	6	1	5
<u>Penicillium oxalicum</u>	6	2	4
<u>Penicillium palitans</u>	6	1	5
<u>Rhodotorula mucilaginosa</u>	6	1	6
<u>Aspergillus terreus</u>	5	4	4
<u>Arthrobotrys sp.</u>	4	1	3
<u>Aspergillus ochraceus</u>	4	3	4
<u>Candida sp.</u>	4	1	2
<u>Dematiaceae</u>	4	2	4
<u>Doratomyces stemonitis</u>	4	2	3
<u>Gliocladium roseum</u>	4	1	4
<u>Phialophora sp.</u>	4	3	3
<u>Rhizopus arrhizus</u>	4	2	4
<u>Syncephalastrum racemosum</u>	4	3	3
<u>Fusarium moniliforme</u>	3	1	3
<u>Fusarium roseum</u>	3	1	3
<u>Penicillium fellutanum</u>	3	2	3

Table 49a. p. 2.

<u>Penicillium variabile</u>	3	3	3
<u>Torulopsis inconspicua</u>	3	1	2
<u>Trichosporon capitatum</u>	3	1	3
<u>Aspergillus sydowii</u>	2	2	2
<u>Candida guilliermondii</u>	2	2	2
<u>Epicoccum purpurascens</u>	2	2	2
<u>Myrothecium verrucaria</u>	2	2	2
<u>Penicillium nigricans</u>	2	2	2
<u>Rhodotorula glutinis</u> var. <u>rubescens</u>	2	2	2
<u>Aspergillus flavipes</u>	2	1	2
<u>Candida humicola</u>	2	1	2
<u>Candida intermedia</u>	2	1	2
<u>Penicillium corylophilum</u>	2	1	2
<u>Penicillium islandicum</u>	2	1	2
<u>Stemphylium</u> spp.	2	1	1
<u>Stemphylium lanuginosum</u>	2	1	2
<u>Stachybotrys atra</u>	2	1	2
<u>Trichosporon fermentans</u>	2	1	2

The following species were observed only once in this series of samples:

Absidia corymbifera, Aspergillus unguis, Botrytis cinerea, Candida mycoderma, C. ?solani, Cladosporium sp., C. herbarum, Cylindrocarpon radiculicola, Gliomastix convoluta, Humicola grisea, Kloeckera apiculata, Leptographium sp., Monotospora humicola, Mucor sp., M. hiemalis, M. racemosus, Paecilomyces sp., P. elegans, Penicillium commune, P. frequentens, P. martensii, P. parvum, P. piscarium, P. purpureogenum, P. stipitatum, P. velutinum, Peyronellea sp., Phialophora fastigiata, Phoma herbarum, Rhizopus sp., R. stoloniferum, Rhodotorula glutinis, R. graminis, R. minuta, Saccharomyces sp., Thielavia sepedonium, Trichosporon behrendii, Trichothecium roseum, Yellow yeasts.

* The plants sampled include: 5 sets from Yellow Springs, 1 set from Columbus, and 1 partial set from Hamilton, all in Ohio.

Table 49b. Fungi observed in isolations from various* sewage treatment systems.

Fungi	Observation Number
<u>Penicillium</u> spp.	131
<u>Moniliaceae</u> spp.	83
<u>Penicillium lilacinum</u>	32
<u>Phialophora jeanselmei</u>	19
<u>Phoma</u> spp.	17
<u>Rhodotorula mucilaginosa</u>	16
<u>Trichoderma viride</u>	16
<u>Geotrichum candidum</u>	14
White Yeasts	14
<u>Trichosporon cutaneum</u>	14
<u>Fusarium aquaeductuum</u>	13
<u>Rhodotorula</u> spp.	13
<u>Penicillium funiculosum</u>	11
<u>Alternaria tenuis</u>	10
<u>Aspergillus ustus</u>	10
<u>Aspergillus carneus</u>	8
<u>Aspergillus niger</u>	8
<u>Aspergillus ochraceus</u>	8
<u>Penicillium janthinellum</u>	8
<u>Aspergillus candidus</u>	7
<u>Aspergillus fumigatus</u>	7
<u>Aspergillus terreus</u>	7
<u>Aspergillus versicolor</u>	7
<u>Candida krusei</u>	7
<u>Cladosporium cladosporioides</u>	7
<u>Aureobasidium pullulans</u>	6
<u>Torulopsis candida</u>	6
<u>Chaetomium globosum</u>	5
<u>Epicoccum purpurascens</u>	5
<u>Aspergillus flavipes</u>	4
<u>Fusarium oxysporum</u>	4
<u>Fusarium roseum</u>	4
<u>Mucor fragilis</u>	4
<u>Penicillium chrysogenum</u>	4
<u>Penicillium variable</u>	4
<u>Saccharomyces cerevisiae</u>	4
<u>Candida humicola</u>	4
<u>Candida tropicalis</u>	3
<u>Cephalosporium</u> spp.	3
<u>Fusarium</u> spp.	3
<u>Mucor alternans</u>	3
<u>Mucor plumbeus</u>	3
<u>Penicillium purpureogenum</u>	3
<u>Phoma herbarum</u>	3
<u>Scopulariopsis brevicaulis</u>	3
<u>Rhodotorula glutinis</u>	3
<u>Trichosporon pullulans</u>	3
<u>Absidia corymbifera</u>	2
<u>Aspergillus flavus</u>	2
<u>Aspergillus sydowii</u>	2
<u>Aspergillus tamarii</u>	2
<u>Candida intermedia</u>	2
<u>Candida ?solani</u>	2
<u>Fusarium solani</u>	2
<u>Gloeosporium</u> sp.	2
<u>Paecilomyces</u> sp.	2

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<u>Penicillium herquei</u>	2
<u>Sporotrichum sp.</u>	2
<u>Trichosporon sp.</u>	2
<u>Zygorhynchus moelleri</u>	2

The following species were observed only once in this series of samples:
Aspergillus clavatus, Curvularia lunata, Dematiaceae sp., Diplodina sp., Gliocladium catenulatum, Gliomastix convoluta, Monilia sitophila, Penicillium claviforme, P. corylophilum, P. corymbiferum, P. expansum, P. javanicum, P. raistrickii, P. rolfsii, P. rubrum, P. rugulosum, P. vermiculatum, Phialophora sp., Rhizopus arrhizus, Scopulariopsis asperula, Stemphylium consortiale, Stilbellaceae sp., Torulopsis dattila, T. famata, T. glabrata.

* Plants summarized in this table include: 42 samples from 6 plants: 1 set from each Hydrocarbon oxidation experimental filter, Taft Center; Ithaca, New York; Hilton-Davis Chemical Co. lagoons, Milwaukee Sewage Treatment Plant, Wisc., Westby, Wisconsin, furrow-type treatment system; and a series of soils used in an experimental septic tank study at Taft Center.

G. Predacious Fungus Behavior in Activated Sludge Systems

The efficiency of biological processes in the removal of complex chemicals from water depends at present on the development of suitable microbial populations. In a recent investigation of the persistence of nitriles in water, the functioning of several laboratory activated sludge units was apparently impaired by a predacious fungus, Zoophagus insidiana. This paper describes the events as observed.

Study conditions. One activated sludge unit served as a stock tank; four received a feed mixture of settled sewage and nitrile in various proportions. The four test units had been acclimated to a specific nitrile over a period of several weeks using a semi-continuous feed. Two additional activated sludge units, designated as Units 6 and 7, were operated on a fill-and-draw basis for preparation of an activated sludge seed. Each of the seven units had demonstrated the ability to produce a clear, highly stabilized effluent, with suspended solids usually ranging from 2 to 15 mg/l and a BOD of less than 10 mg/l. The nitrile sludges generally required withdrawal of 10 to 30 per cent of the solids per week to maintain a mixed liquor-solids proportion of about 2,000 mg/l. Microscopic observation of the sludges showed a mixed population with variations apparently associated with the particular nitrile feed.

Appearance of fungus. These performances changed drastically within a few days. Settling characteristics of all units changed at about the same time. The stock unit and Units 6 and 7 showed the most serious changes.

Adiponitrile and acrylonitrile units showed intermediate effects and lactonitrile and oxydipropionitrile units were affected least. Effluent suspended solids ranged from 20 to 90 mg/l. Mixed liquor solids were reduced concurrently by as much as 1,000 mg/l in some units within one week.

Superficial examination of units showing the most obvious alteration revealed gray to white filamentous masses similar in appearance to Sphaerotilus. Microscopic examination showed the "wild" growth to be present in all units but the masses were large enough to be seen in only three. The abundance of the feathery growth and the obvious imbalance of the biota in the activated sludge systems coincided with the poor performance and required further work in identification, behavior, and control techniques.

It has long been considered that an activated sludge is maintained in its most favorable condition as long as such predatory organisms as flagellates, ciliates, amoebae and rotifers are present to control the growth of the bacteria which actually remove, by various degradation processes, the impurities of sewage which are present in solid, colloidal, or dissolved states. Microscopic examination of the grey, feathery floc showed an interesting type of true fungus and demonstrated immediately the probable source of the trouble. In addition to occurring as visible white streamers, the fungus forming this floc was found throughout the sludge.

The fungus found in the "sick" activated sludge was a predatory fungus, Zoophagus insidians, whose diet consists of rotifers, in this case of the genus Monostyla. In the seven activated sludge jars under consideration,

the principal predator of the bacterial population was Monostyla. In other activated sludge experiments in which sewage from the same source was used, principal organisms included nematodes, stalked ciliates such as Epistyla, and other organisms, while Monostyla was rare or absent. Zoopagus was not evident in these tanks.

The Predacious Fungi

Fungi which possess the predacious habit were known as early as 1852 although it was not realized that they were not ordinary molds until their predacious habit was discovered 75 years ago (Duddington, 1957b). Drechsler, in the United States, has been interested in this type of fungus since 1934, and Duddington, in England, since 1940. More than a hundred species of predatory fungi have been reported, mainly since 1934. These fungi are of interest, from an economic point of view, principally because of their potential ability to aid in the control of tuber diseases of potato, and root diseases of beet and cereal crops which are caused by excessive numbers of parasitic nematodes in the soil.

Zoopagus. The predatory fungus which was devouring Monostyla in the activated sludge units being studied is a common species, Zoopagus insidians Sommerstorff. It was first observed in Austria in 1911 (Duddington, 1957b). The original collection was made in relatively clean water in which the alga Cladophora was growing. A species of Monostyla was the rotifer upon which it was feeding in the original collection. Since that time it has been reported from Bulgaria, Yugoslavia, France, England, New York, and

Maine. Fast-moving species of microscopic animals are too powerful to be held by it. In culture dishes it captures various types of browsing rotifers, and in a short time can eliminate whole populations.

Action of predatory mechanism. The action of the predatory mechanism is interesting. From the time of the germination of the gemmae or conidia (Figure 20), spine-like or peg-like capturing organs (Figure 21) are produced along the mycelium. At the tips of these organs a mucilaginous material is secreted. When the browsing rotifer reaches the tip of the capturing organ (possibly stimulated by the discharge from the capturing organ of some as yet unidentified substance or by the presence of bacteria which form the food of the rotifer) the mucilaginous material, which is secreted in sufficient abundance, causes the rotifer to adhere to the capturing organ. From this organ a poorly developed appressorium or haustorium is then formed and from this a mycelial thread grows into the "mouth" of the rotifer and throughout its body (Figure 22). When the protoplasm in the body of the rotifer has been consumed, the cytoplasm in the haustorium appears to be returned to the mycelium. The shell of the rotifer, with whatever fungal tissue is left, is discarded. The capturing organ then is apparently ready to trap another rotifer. Under certain conditions, not observed in this study, zoosporangia are formed in the shell of the rotifer and the resulting zoospores are released through its wall.

Terminology. In previously published reports of this fungus, the terms "conidia" and "gemmae" are used as relating to rarely produced structures. Since the fungus has been classified by many workers in the



FIGURE 20

Several hyphae of Zoophagus insidians with free and captured individuals of Monostyla. A normal individual of Monostyla appears in upper right; others have been captured and are in various stages of digestion. Note cluster of conidia and a conidiophore in center.



FIGURE 21

Hyphae of Zoophagus insidians showing capturing organs. Note captured Monostyla and the exhausted shell of a second one.

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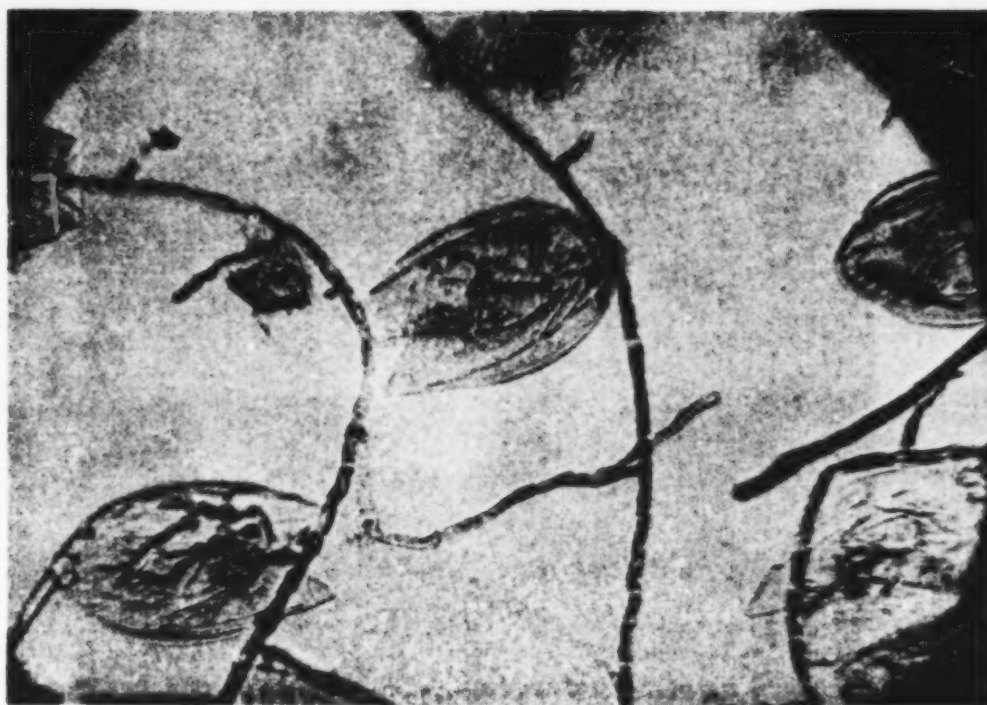


FIGURE 22

Two filaments of Zoophagus inisidians. The one on the left has trapped a Monostyla on each of two capturing organs. Mycelium has entered the upper Monostyla and has reached its maximum development. Digestion of the lower one is about completed.

Pythiaceae (and more recently in its own family, the Zoopagaceae) it is traditional to refer to such non-motile hyphal segments which act as reproductive organs as gemmae. However, in the material under consideration, some of the capturing organs develop a cap at their tips on which are produced two to eight elongate conidiospores (Figure 20). These may technically be considered as gemmae since they are non-motile hyphal branches, but because of their abundance in our material and because of their method of production and liberation, they are better considered as conidiospores. From the outset these conidiospores are filled with dense cytoplasm which may be highly granular. They are produced in a crown on the tip of the modified capturing organ and are cylindrical in shape. Apparently their method of discharge, following abstriction, is a mechanical breaking off as a result of maturity and the continuous activity forced upon them in the aeration process. At maturity these spores may reach 100 to 120 μ in length and have a width of 5.5 to 10 μ . Following release they may elongate to as much as 170 to 200 μ and become septate before production of germ tubes on the terminal cells and capturing organs on the other cells. Germination may occur at both ends and at each cell, and, as soon as the germ tubes are produced, the capturing organs are formed on the hyphae as well as on the cells of the spore.

Observations. Techniques used in the laboratory, which included both direct observation and rapidly prepared temporary or semi-permanent mounts, permitted observation of all the pertinent structures with little effort and small loss of material or detail. In direct observation, a drop of sludge containing the organism was placed on a slide, a cover slip

was added, and observations were made on the actively moving materials. For temporary mounts, a drop of methylene blue could be added so that cells and some cell contents could be differentially stained. For semi-permanent mounts, a drop of sludge containing fungus material with associated rotifers, or a floc composed of the fungus and accompanying organisms, was placed on a glass slide and a drop of lacto-phenol-acid fuchsin or lacto-phenol-cotton blue was added. A cover glass was placed on the stained portion of the material and excess material was removed by blotting with toweling or other convenient absorbent material. The hyphae of the fungus were stained immediately with no apparent shrinkage. The hypha which penetrated the body of the rotifer stained more slowly, usually in less than a half hour in acid fuchsin (over-night in cotton blue), and could be seen to good advantage. For semi-permanent storage, the cover glass could be ringed with balsam or other sealing preparations.

In this investigation zoospore formation in the mycelium within the rotifer body has not so far been observed, probably because the growth in the activated sludge was not readily adapted to this method of spore production and because conidiospore formation was adequate for dispersal. During the course of the experiment, with its attendant difficulties, starvation, which is one method of triggering spore formation in the fungi, was not attempted. Rotifers were captured by the fungus when the substrate included acrylonitrile as well as in sludges containing no nitriles. Conidiospores were produced in 2's or 3's instead of in clusters numbering up to eight.

Chlorination of cultures. The long period of sludge acclimatization required for nitrile assimilation made it necessary to save the activated sludge cultures if possible. Since chlorination has been used to control wild growth in activated sludge systems on numerous occasions, it was accordingly tried here. Sodium hypochlorite solution was applied to the mixed liquor, batchwise, in daily doses of 10 mg/l as chlorine for two days. Then the dosage was cut to 5 mg/l for 5 additional days.

The first two doses of chlorine resulted in a marked reduction of Zoopagus and Monostyla. The mechanism of reduction was not determined. A small portion of the mycelium floated out with the effluent as scattered fragments. Chlorination inactivated most of the Monostyla. Zoopagus may have been directly affected by chlorine. A large increase in active copepods occurred following chlorination. Although the predatory fungus virtually disappeared after the first two chlorine applications, five additional chlorine doses were applied mainly to destroy mycelium developing from remaining spores. All units were fed throughout chlorination in the original sequence.

Biological effects. Chlorination had a marked effect upon other biological forms in the units. Protozoans and other forms of life increased in variety. Copepods appeared in large numbers. Stalked ciliates, flagellates, and nematodes, relatively scarce in the sick sludge, became numerous. Production of oxidized nitrogen fell off about 95 per cent after several days of chlorination. Nitrification had not decreased during the period of wild fungal growth. During the middle of the second week after chlorination, one unit showed an increase in effluent oxidized nitrogen.

The remaining four showed a similar increase four days later. Apparently, chlorination resulted in a major decrease in the population of nitrifying organisms. Their slower regeneration resulted in a much longer recovery time than for other active organisms.

Sludge condition. Activated sludge effluents remained in poor condition during the chlorination period. Suspended solids ranged from 20 to 40 mg/l. Mixed liquor solids continued to decrease slowly. Only one to two days were required after discontinuance of chlorination to obtain a clear effluent. Mixed liquor solids began a rapid rise about two weeks after chlorination.

Effect on nitrile-consuming organisms. Nitrile assimilation was undetermined during the period of upset and recovery because of the nitrogen imbalance resulting from sludge destruction. As soon as oxidized nitrogen in the effluent exceeded nitrogen entering in sewage, nitrile nitrogen oxidation became evident, occurring in one test unit during the second week after chlorination and, in the three remaining units, during the third week after chlorination.

It was apparent that nitrile-consuming organisms were not completely destroyed by the upset or by subsequent chlorination. Although up to 11 weeks were required to acclimate a new sludge to nitrile oxidation, all four test units were effectively metabolizing nitriles within three weeks after chlorination.

Conclusions. Chlorine dosage used to correct the wild growth apparently was greater than necessary to accomplish the desired effect. Fewer applications at a lower concentration probably would have destroyed the fungus without seriously affecting nitrifying organisms.

In Units 6 and 7, which were not chlorinated, Zoopagus was still evident at a time when the chlorinated units were producing good effluents. The mold was disappearing slowly as if running out of food or because of the large increase in copeopods. It is likely that the population would have been restored eventually in the test units without treatment but good effluents were obtained two to four weeks earlier in the chlorinated units.

H. Enumeration of Yeasts in Sewage Treatment Systems and Polluted Water

An indicated number (IN) technique was devised for the enumeration of yeast populations in sewage and polluted waters. To test the technique, three sets of samples were obtained from the Dayton, Ohio sewage treatment plant in May and June, 1962 and in April, 1963. One set of samples was obtained from the Hamilton, Ohio sewage treatment plant in April, 1963, and one set of river water samples from the Columbia River, Oregon-Washington, in January, 1963.

At Dayton, samples were obtained of raw, settled, filtered and final sewage, settled, digested and drying sludge, and scrapings from trickling filter stone. At Hamilton, samples were obtained of raw, settled, aerated, secondary settled and final sewage, settled and digested sludge, and return sludge. Six of these samples are comparable, but secondary settled sewage was not sampled at Dayton, and return sludge is not exactly comparable with trickling filter stone scrapings. In the trickling filter the population remains in place as the sewage flows over it, in the activated sludge tank the population moves with the sludge through the aeration

tank. Samples from the Columbia River were obtained by Portland regional office personnel of the Public Health Service. Samples were returned to Cincinnati the day of collection (by air mail from Portland) and refrigerated until they could be processed. Processing is described in Chapter II.

Results

Data from these samples are presented here. The samples represent stages in two phases of two types of biological treatment processes and grab samples from a flowing river, and not portions of any single batch of sewage or type of pollution.

Table 50 shows the results of the isolations from the set of samples collected at Dayton, April 26, 1963. They are based on only the 1:10 dilution. When scanning the flasks or first plates streaked with settled yeast cells from those flasks, if only white yeasts are present, only 10 cells per ml of sample can be reported unless strikingly different colonies appear on the plates, if red yeasts are also present, at least 20 cells per ml can be counted; if the results of colony identification are completed this value can be increased to ten times the number of identified species. In the table the scanning value is separated from the species value by a dash.

This kind of information can be supplemented with the oven-dry weights of the growth obtained after 64 hr on the shaker. Table 51 shows that at the 1:10 dilution good growth was obtained with each level of glucose. Some growth occurred at all dilutions. When this growth is

Table 50. Indicated number of cells per milliliter of sample based on 1:10 dilution (Total species 18).

Medium	Sample habitat							
	1	2	3	4	5	6	7	8
Raw Sewage		Settled Sewage	Filtered Sewage	Final Sewage	Settled Sludge	Digested Sludge	Drying Sludge	Trickling Filter slime
YNB + 1% glucose	20-70	10-50	20-60	10-30	10-30	10-10	10-30	10-80
YNB + 20% glucose	20-60	20-60	10-80	10-60	10-20	20-30	20-20	20-110

Note: Growth from samples collected April 26, 1963. Value preceding dash based on inspection of flask or initial streaked plates; value following dash based on number of species isolated from each flask.

Table 51. Dry weight, in milligrams, of growth obtained after 64 hours in shaker in YNB medium*

Amount Glucose	Dilution	Sample Habitat							
		1 Raw Sewage	2 Settled Sewage	3 Filtered Sewage	4 Final Sewage	5 Settled Sludge	6 Digested Sludge	7 Drying Sludge	8 Trickling Filter Slime
1%	1:10	186.3	194.3	213.2	220.1	187.0	195.6	11.2	184.6
	1:100	178.2	193.5	82.0	108.2	212.3	19.5	22.8	161.6
	1:1000	192.3	5.0	35.2	13.1	10.5	10.1	16.0	168.7
	1:10,000	75.4	179.5	7.4	13.9	17.5	7.2	7.5	180.0
	1:100,000	90.9	34.5	12.7	11.1	10.6	15.4	7.9	82.2
20%	1:10	255.0	326.1	257.0	248.9	276.8	291.8	244.4	272.8
	1:100	165.1	299.2	118.5	135.2	68.2	92.1	119.7	307.0
	1:1000	111.1	119.6	47.4	44.9	130.0	354.3	52.5	309.3
	1:10,000	34.6	64.0	57.4	70.3	97.8	191.0	53.5	302.8
	1:100,000	110.3	44.6	66.0	92.7	124.1	643.9	105.7	83.3

* Indicated number by inspection is represented by dilution number. Indicated number based on identified species is usually higher (See Section 3 of Table 52).

Survey of April 26, 1963.

streaked on agar plates, growth of yeasts is demonstrated in most flasks. Often more than one type of colony appears on each plate. Strains are identified after the cultures have been purified (Table 52). The number of species in each sample on each medium at each dilution is represented by the total of the digits in each of the values given for the numbers of cells present. It is simpler to note the number of different types of colonies in each set of isolates; the numeral 1 would represent a single type or the numeral 2, two types of yeast colonies. Two strains of one species may have differing colony types, while colonies of two or more species may look alike. Hence, with a mixed plate, the error may be conservative or liberal and the ability to pick colonies of all species present is not perfect.

Species obtained from shaken flask cultures (the IN method) differ from those obtained from still culture bottles (the MPN method) because some species grow well without aeration while others do not. The surface of still cultures may become covered with mold mycelium that restricts oxygen diffusion

Discussion

The indicated number technique is adaptable for estimating numbers of yeast cells in the various types of materials from a sewage treatment plant. Shaken liquid culture is the preferred technique using both 1% and 20% glucose in a yeast-nitrogen-base solution. If more than one medium for each type of sample is used, more species can be obtained. A pattern

Table 52. Minimal number of yeast cells per milliliter of original sample based on number of species isolated. (Total species - 29)

Date	Medium	Sample Habitat*							
		1	2	3	4	5	6	7	8
		Raw Sewage	Settled Sewage	Filtered Sewage	Final Sewage	Settled Sludge	Digested Sludge	Drying Sludge	Trickling Filter Slime
Indicated Number									
1962									
May	YNB-1% glucose	10,210	110,210	1,010	110	110,310	110,010	110	202,040
June	YNB-1% glucose	100,230	102,110	200,010	210,110	103,130	1,210	10,100	100,310
May	YNB-20% glucose	12,410	1,110	100	120	110,220	111,120	10	11,020
June	YNB-20% glucose	201,020	200,110	100,110	201,120	112,200	10,020	1,210	121,110
Most Probable Number									
1962									
June	YNB-1% glucose	211,210	1,240	10,210	20	103,210	1,210	10,010	112,200
June	YNB-20% glucose	202,250	110,120	11,230	110,120	342,200	212,300	10,210	220,200
Indicated Number									
1963									
April	YNB-1% glucose	111,220	10,310	11,130	120	120	10	1,020	133,100
April	YNB-20% glucose	101,220	1,320	1,520	200,220	1,010	210	110	132,320

* From Dayton, Ohio, sewage treatment plant.

runs through species lists from different sets of samples within one season, but there appears to be no uniform distribution of one group of yeast species compared with another unless many techniques and media are used.

Oven-dry weights of tissue and turbidometry of developed cells can be used to supplement species lists, but in general populations can be estimated visually as to quantity but not as to species composition. Dry weights may reflect the reaction of one or another species to the changed culture conditions and not the original population levels.

Within the sewage treatment system the yeast population is probably introduced with the sewage, but it increases some as the sewage passes through the plant, and is maintained in the slimes on the surface of the stones of the trickling filters. Portions of such populations reach the sludges, survive the digestion process, and remain in the air-drying sludges on the drying beds. The populations differ from one time to another, but contain species that appear to be constant throughout the process. Overlapping segments of the population are obtained by using different media and methods.

A composite list of species isolated shows that the more techniques used per sample, the more complete is the list obtained. This observation is based on the June, 1962 samples where techniques were used to obtain both the IN and the MPN values. These samples had more species, and more species were present in a larger number of samples than on the other sampling dates. This is probably because there were more opportunities for yeast colonies to appear.

The results of the observations do not show a continuous pattern of increase, reduction, or uniformity of populations. Rather, there is a lack of uniformity within the three sets of samples (Table 52). This appears to be associated with the ability of one species or another to develop on a medium, or the presence or absence of a species in the milliliter of sample or its dilutions. It may also be due to variations within the populations in incoming sewages modified by addition of organisms from sludges recirculated within the plant.

In Table 52 the large numbers of yeast cells recovered from various stages of a sewage treatment process give at least a superficial indication that yeasts are effective members of a population in the biological treatment of sewage. In general, even if species are of fecal origin, sewage or organic enrichment produces an adequate habitat for growth in the process of which some of the polluting substances are removed.

VII. Waste Stabilization Ponds

A. General

Introduction

Waste-stabilization ponds are planned, basin-type installations used for the partial or nearly complete treatment of sewage or industrial wastes; pollution in effluents from such ponds is much less than that in untreated waste waters. Because it is a less expensive installation, when land is available, it is suited to the needs of a small community, institution, or industry. Bacteria and algae are among the organisms which act on the sewage passing through such a system. Although the presence of fungi and protozoa may be assumed, until now fungi have received little attention.

The waste stabilization pond system studied was located on a penal honor farm maintained near Lebanon, Warren County, by the Ohio Department of Welfare and Correction. Plans for the system were laid in the summer of 1955. Following a study of the soil in September, 1955, four 1/2-acre ponds and one 2-acre pond were constructed by the excavation of 45 to 60 cm of soil from an alluvial bench to form dikes which were compacted to retain water. The undisturbed soil is Genessee silty clay with a well-developed granular structure in the upper 30 cm and a nut structure in the next 30 cm. Below 60 cm there is a larger percentage of clay, and the structure is more columnar. The alluvial material in the valley of Shaker Creek is partly glacial in origin and overlies bedrock of Ordovician limestone. A diagram of the system is presented in Figure 23.

On July 22, 1957, soil samples from eight points on the bottom of unfinished ponds were processed for mold populations. Most of the samples

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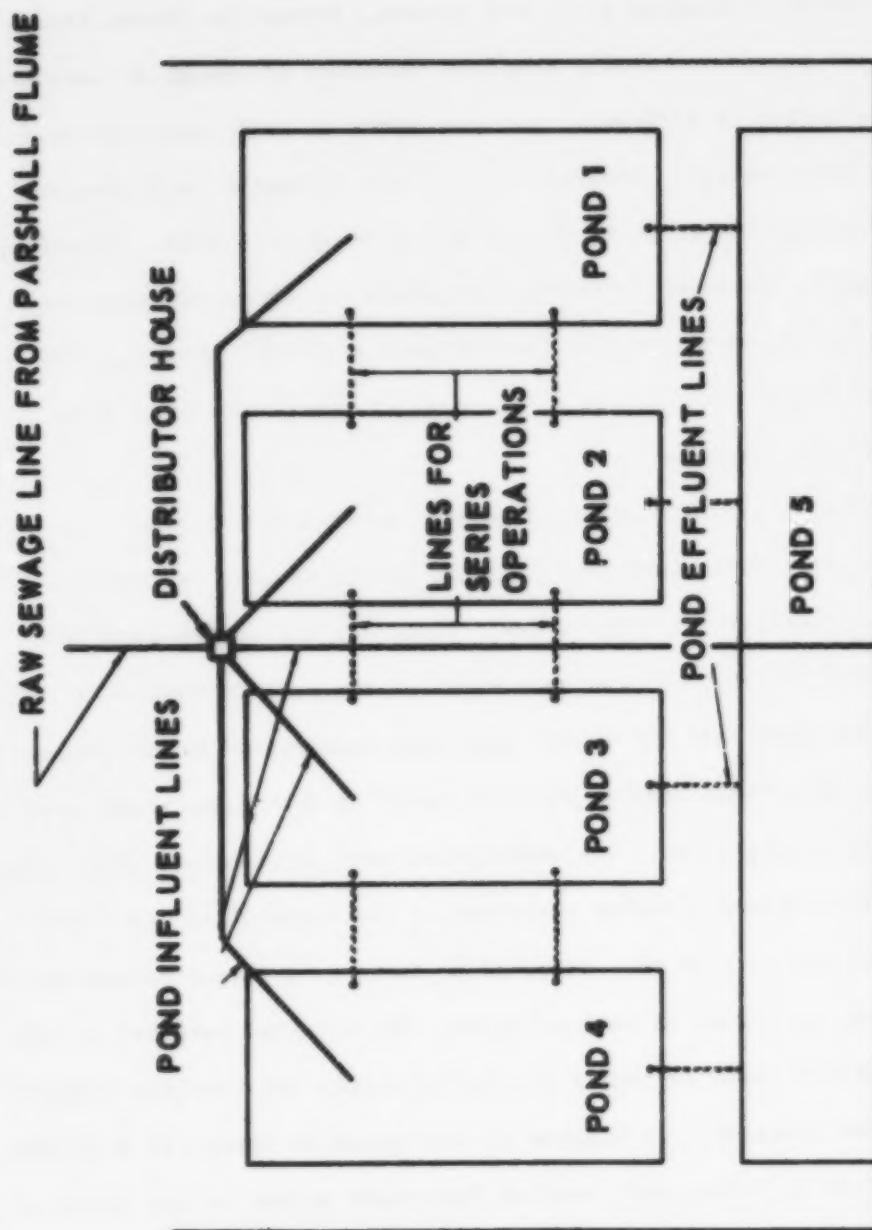


FIGURE 23. Plan view of Lebanon ponds and piping arrangement.

were from clays which underlay the surface soils previously planted in corn or other garden crops. In June, 1958, during a preliminary trial, use of the ponds for sewage disposal was discontinued because of extensive seepage. Soil and seepage characteristics of the ponds were restudied in the autumn of 1958. As a result, the ponds were lined with various materials to improve their ability to hold water. Soil samples from similar but not identical locations to the 1957 series were taken on February 9, 1959. These demonstrated mold colonies in larger numbers than those from the 1957 series. A detailed description of these ponds is given by Horning et al. (1964).

In an attempt to improve the ability of the soils to hold water, the following treatments were used: Pond 1, which was lined with a thin sheet of plastic, retained water at the desired level. Pond 2, lined with bentonite clay, retained water fairly well. Pond 3, which was unlined, did not retain water at the required level to the end of the project. Pond 4, which was lined with bentonite clay, did not retain water except during periods of heavy precipitation. Pond 5, the large 2-acre pond designed as a second holding pond for further biological treatment, received water that had passed through the other four ponds; it retained little water during the entire study. Effluents from Ponds 1 and 2 produced marshy areas in Pond 5, but, except during periods of high precipitation, no standing water was observed.

Overflow water from Pond 5 was to have been piped under an adjacent corn field to Shaker Creek, but no such overflow developed. Fungus sampling stations were set up on Shaker Creek at a point above which raw wastes were received from the dormitory kitchen, and at a point where the

effluent from Pond 5 should have entered the creek. It is difficult to say whether the seepage from Ponds 2, 3, 4, and 5 actually traveled to the latter point or whether it reached the creek at some lower point or points.

The ponds were designed so that adjacent ponds could act as secondary holding ponds for effluents from their neighbor. At first, Pond 3 overflowed into Pond 4, which also could have received raw sewage from the distributor that fed Ponds 1, 2, and 3; however, use of Pond 4 was discontinued when it became evident that even with the extra bentonite clay lining the pond could not hold water. The raw sewage was then fed into Ponds 1, 2, and 3, each of which overflowed into Pond 5, whose contents seeped into the adjacent soils (cf Horning et al (1964)).

The design characteristics and the hydrology of the two usable man-made ponds (Ponds 1 and 3) are presented by Horning et al (1964). Similar data for the other habitats sampled are not available. The amount of water received and lost by the ponds for each of the four seasons is given. Most of the water routinely received by the ponds was sewage; it formed the habitat and supplied many of the disseminules of the fungus population; thus the hydrologic data are representative of the habitat for the populations described here. Atmospheric temperature and precipitation data are available in Weather Bureau reports for Cincinnati and Dayton stations. Some local temperatures are given by Horning et al (1964).

Methods

Certain data are basic to the understanding of the performance of a sewage-treatment system; usually these are taken from samples obtained

within the system. A temporary field laboratory was set up on one of the dikes near the distributor through which the sewage entered the ponds. The study was divided into four 4-week sampling periods, (spring, summer, autumn, winter) during which the field laboratory was manned 24 hours a day by technical personnel from the Robert A. Taft Sanitary Engineering Center, and by men from the dormitory. Samples for bacteriological, chemical, and physical analyses were collected every 2 hours and analyzed immediately, or they were sent to the Center for more detailed analysis. The study of fungal populations was made in parallel with this study but to avoid disturbance of the ponds during bacteriological analyses, samples were obtained during the week preceding the two-week period and the week following this period, when possible. Spring samples were taken on April 1 and 18, 1960, summer samples on July 25 and September 9, 1960, autumn samples on October 22 and November 7, 1960, and winter samples on February 24 and March 13, 1961. Results on other aspects of the study appear in Morning et al (1964).

Habitats from which fungi were sampled are identified in Table 53. One vial of a grab sample from each habitat on each date was usually collected between 9 and 10 o'clock in the morning and these were processed on the same day in the Fungus Studies Laboratory of the Sanitary Engineering Center. Samples of liquids and sludges in the ponds were taken in coffee cans nailed to one end of a 10-foot pole. A portion of each such canful of material was poured into the vial.

Techniques of isolation of fungi and the media used have been described in detail (Cooke, 1962, 1963; Cooke and Matsuura, 1963, Chapter II). For

Table 53. Habitats and Numbers of Fungi Isolated - Lebanon Waste-Stabilization Ponds

Habitat Number	Number of Samples Obtained	Habitat Description
1	8	Undisturbed soil in adjacent fence row
2	8	Rav Sewage
19-20*	8	Pond 5, Random samples from bottom of pond, swampy
33-34*	7	Soil samples from muskrat dens on dike of Pond 1
3	8	Pond 1, sludge near influent pipe
4	8	Pond 2, sludge near influent pipe
5	8	Pond 3, sludge near influent pipe
14	8	Pond 4, sludge near influent pipe
6	8	Pond 1, sludge near effluent pipe
7	8	Pond 2, sludge near effluent pipe
8	8	Pond 3, sludge near effluent pipe
15	8	Pond 4, sludge near effluent pipe
9	8	Pond 1, mid-pond water
10	8	Pond 2, mid-pond water
11	8	Pond 3, mid-pond water
16	4	Pond 4, mid-pond water
12	7	Pond 1, effluent
13	5	Pond 2, effluent
39	3	Pond 3, effluent (not always available)
21	7	Pond 1, scum, N.E. corner of pond
22	8	Pond 2, scum, N.E. corner of pond
23	7	Pond 3, scum, N.E. corner of pond
24	3	Pond 4, scum, N.E. corner of pond
17	8	Pond 5, mud in rill carrying effluent from Pond 1
18	8	Pond 5, mud in rill carrying effluent from Pond 2
40	5	Pond 5, mud in rill carrying effluent from Pond 3
25	8	Shaker Creek, upstream, water
26	6	Shaker Creek, upstream, water and sediment in riffle
27	6	Shaker Creek, upstream, water and sediment in pool
28	7	Shaker Creek, upstream, wet bank soil
29	7	Shaker Creek, downstream, water
30	6	Shaker Creek, downstream, water and sediment in riffle
31	7	Shaker Creek, downstream, water and sediment in pool
32**	6	Shaker Creek, downstream, wet bank soil

*Samples from Habitats 19 and 20, 33 and 34, combined.

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Spring	Season					Average Number of Colonies	Average Number of Species			
	Summer		Autumn		Winter					
	7-25-60	Sampling Dates 9-7-60	10-22-60	11-7-60	2-24-61			3-13-61		
4-1-60	4-18-60	62	28	38	69	84	49	89	59	15.6
			Ratio of Colonies in each habitat to those in undisturbed soil							
49	6.5	4.19	1.42	0.63	5.8	9.5	40.8	2.25	510	4.0
	1.9	25.8	2.21	68.4	136.2	154.8	24.5	39.3	3,800	11.6
	42.9	25.8	13.9	105.3	----	50.0	13.9	30.3	2,500	12.1
	.16	.27	.14	.47	.14	.24	4.08	3.03	68	10.5
	.59	.32	.26	.66	.083	1.02	5.92	8.65	150	7.0
	1.75	10.5	.21	.90	.38	1.02	2.86	79.8	900	5.8
	.67	.94	.50	5.26	5.8	33.3	75.5	98.9	2,000	9.5
	.65	1.18	.23	.68	.22	1.05	2.45	2.25	71	8.8
	4.7	2.41	.31	1.05	.62	.56	.88	2.81	110	4.4
	3.5	2.9	2.64	1.29	.58	1.55	4.7	5.51	170	5.3
	2.04	2.74	8.21	29.6	333.3	119.1	61.2	134.8	6,200	7.5
	.73	1.18	.64	868.4	1.06	3.2	3.7	21.3	4,400	6.8
	4.9	22.6	7.86	1.76	.29	.74	4.5	29.2	600	5.3
	3.27	19.4	3.18	4.74	1.88	2.62	6.7	8.76	390	5.8
	2.86	.14	3.32	----	----	----	----	12.4	340	7.0
	2.65	40.3	.89	----	----	----	----	7.87	530	3.8
	5.9	----	4.64	1.97	.87	----	5.1	1.69	230	6.0
	----	----	----	6.32	----	----	7.3	----	200	4.4
	1.02	8.5	.46	6.32	2.32	----	4.08	----	860	8.0
	61.2	37.1	6.79	21.8	49.3	4.3	7.8	4.83	1,300	7.6
	2.65	6.1	5.0	8.95	8.4	38.9	1.29	----	590	9.7
	3.06	2.1	11.4	6.32	34.8	4.4	1.02	----	200	4.7
	42.9	36.5	35.7	5.0	----	41.7	6.5	30.3	1,800	10.1
	46.9	41.9	71.4	16.3	55.1	53.6	3.7	56.2	2,600	12.9
	----	----	----	0.5	78.3	14.3	15.7	66.3	2,700	12.2
	.08	.019	.028	.042	.203	.36	.204	.79	16	6.5
	1.37	9.68	3.9	15.3	11.7	14.3	----	----	560	11.5
	.22	11.8	3.25	1.63	.203	6.55	----	----	240	9.7
	26.5	59.7	27.5	102.6	97.1	42.9	6.94	----	2,800	12.3
	2.86	6.45	.82	50.0	4.35	7.14	1.76	----	490	6.9
	4.29	5.32	7.5	18.4	13.04	20.2	----	----	580	13.8
	5.3	4.52	4.64	5.79	8.55	7.02	.29	----	300	15.1
	32.7	17.7	29.6	22.6	173.9	47.6	----	----	3,400	14.8

Table 53. Footnote ** Under No. 32.

35. Shaker Creek. LD - scum from backwater at habitat 32.
LG - Hydrodictyon net below Habitat 32.
36. LG - Lemna mat from point below habitat 32.
37. Spri freshet below Habitat 32.
38. LF - Hydrodictyon net on pool below habitat 32.

each of the media or technique mentioned here, the inoculum used was a 1:10 dilution of the original sample shaken for 1/2-hour on a rotary shaker at about 150 oscillations per minute. This was diluted again as required to obtain colonies on pour plates adequate for counting and picking purposes.

For purposes of colony counting and picking of mold and yeast colonies for further study and identification, pour plates in replicates of 5 were prepared for all 10 sets of samples with neopeptone - dextrose - rose bengal - Aureomycin agar; they were incubated for 7 days at room conditions of temperature and light. For the study of yeast populations, enrichment of the latter 8 sets of samples were prepared with yeast-nitrogen-base (YNB-Difco) in liquid culture in paired flasks, in one of which 1% dextrose was used, and in the other, 20%. After 64 hours on the shaker, growth was allowed to settle and then was streaked on yeast extract - malt extract - dextrose agar. After 3 days on the laboratory bench, colonies were picked or cultures purified. From the same flasks, molds were recovered on agar and purified, and the data were added to observation values obtained with the agar plates. For the last 5 sets of samples, in addition to the enrichment cultures described above, liquid media were prepared in which to yeast carbon base (YCB-Difco) were added 1% $(\text{NH}_4)_2\text{SO}_4$, NaNO_2 , and KNO_3 , and to YNB were added 1% glucose, galactose, sucrose, maltose, lactose, and ethanol. A solution of vitamin-free yeast base (VFYB-Difco), and a mixture of YNB and YCB were also used. For the isolation of possible aquatic fungi from the last 5 sets of samples, a portion of the 1:10 dilution of the original sample was added to a Petri

dish in which had been placed halved hemp seeds which had been autoclaved. Soil samples collected on September 6, 1960, were placed in sterile Petri dishes, sterile distilled water was added, and short strips of wool cloth placed on the soil. Warcup soil plates were prepared using the same soil samples.

Colony counts resulting in semiquantitative data were possible only on the pour plates. Other media used for isolation of fungi yielded only qualitative data and a lengthened list of species recovered from these habitats. Observation values were obtained by summarizing colony counts, species noted on other than pour plates, and recoveries from liquid cultures.

Results

Difficulties arise from interpreting results from the above methods. Colonies may have arisen from a single spore or hyphal fragment, or from a clump of several spores or hyphal fragments, making population density estimates risky. Selective media favor some species and suppress others, making recognition of all species present difficult. Because of the great variety of species of fungi appearing when qualitative techniques such as these are used, and because of the method of counting plates when the ~~total~~ number of colonies appearing on five plates is averaged, it was not practical to complete a statistical analysis of these populations. In addition, all isolation techniques were not used each time the samples were taken. A composite list of species taken from all media totals 212 species; it is presented as Table 54.

Table 54. Species of Fungi observed in study of Lebanon Waste Stabilization Ponds

Species	Observation Number
Moniliaceae (unidentified)	798
<u>Mucor hiemalis</u>	782
<u>Penicillium</u> spp.	641
<u>Geotrichum candidum</u>	468
<u>Trichoderma viride</u>	426
<u>Candida krusei</u>	364
<u>Fusarium oxysporum</u>	304
<u>Rhodotorula mucilaginosa</u>	246
White yeasts (unidentified)	229
<u>Phoma</u> spp.	227
<u>Rhodotorula glutinis</u>	180
Dematiaceae spp.	150
<u>Phialophora jeanselmei</u>	150
<u>Candida tropicalis</u>	147
<u>Penicillium lilacinum</u>	137
<u>Cladosporium cladosporioides</u>	116
<u>Candida parapsilosis</u>	111
<u>Fusarium aqueductum</u>	110
<u>Saccharomyces cerevisiae</u>	109
<u>Aureobasidium pullulans</u>	104
<u>Penicillium funiculosum</u>	102
<u>Gliocladium roseum</u>	97
<u>Rhizopus</u> spp.	89
<u>Rhodotorula</u> spp.	67
<u>Fusarium acuminatum</u>	62
<u>Epicoccum purpurascens</u>	50
<u>Aspergillus flavus</u>	51
<u>Aspergillus niger</u>	53
<u>Alternaria tenuis</u>	47
<u>Aspergillus ochraceus</u>	44
<u>Candida guilliermondii</u>	43
<u>Aspergillus fumigatus</u>	41
<u>Torulopsis inconspicua</u>	38
<u>Torulopsis glabrata</u>	37
<u>Candida curvata</u>	36
<u>Cephalosporium</u> spp.	35
<u>Aspergillus sydowii</u>	33
<u>Curvularia</u> spp.	33
<u>Torulopsis famata</u>	33
<u>Cryptococcus albidus</u>	32
<u>Cryptococcus laurentii</u>	32
<u>Trichosporon cutaneum</u>	31
<u>Penicillium variabile</u>	30
<u>Paecilomyces varioti</u>	22
<u>Fusarium solani</u>	21
Smut-like	21
<u>Fusarium graminearum</u>	18
<u>Mucor alternans</u>	18
<u>Hansenula saturnus</u>	17
<u>Saccharomyces</u> sp.	17
<u>Monosporium apiospermum</u>	16
<u>Candida intermedia</u>	15
<u>Microthecium verrucaria</u>	15
<u>Candida utilis</u>	14
<u>Hansenula anomala</u>	14
<u>Penicillium purpureogenum</u>	14

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<u>Candida pulcherrima</u>	13
<u>Gliocladium fimbriatum</u>	13
<u>Paecilomyces</u> spp.	13
<u>Penicillium velutinum</u>	13
<u>Pestalotia</u> sp.	13
<u>Torulopsis candida</u>	13
<u>Aspergillus terreus</u>	12
<u>Emericellopsis humicola</u>	11
<u>Endomycopsis javanensis</u>	11
<u>Penicillium herquei</u>	11
<u>Candida humicola</u>	11
<u>Gliocladium</u> spp.	10
<u>Gliocladium deliquescens</u>	10
<u>Trichosporon pullulans</u>	10
<u>Aspergillus clavatus</u>	10
<u>Fusarium moniliforme</u>	9
<u>Torulopsis vanzylii</u>	9
<u>Aspergillus</u> spp.	9
<u>Aspergillus candidus</u>	8
<u>Candida rugosa</u>	8
<u>Cryptococcus luteolus</u>	8
<u>Fusarium conglutinans</u>	8
<u>Aphanomyces</u> sp.	8
<u>Penicillium expansum</u>	7
<u>Penicillium rubrum</u>	7
<u>Pichia membranaefaciens</u>	7
<u>Pythium</u> spp.	7
<u>Saprolegnia</u> spp.	7
<u>Aspergillus carneus</u>	7
<u>Colletotrichum gloeosporioides</u>	6
<u>Penicillium</u> nr. <u>luteum</u>	6
<u>Penicillium martensii</u>	6
<u>Achlya</u> spp.	6
<u>Allomyces</u> sp.	5
<u>Aspergillus tamarii</u>	5
<u>Aspergillus terreus</u> var. <u>floccosus</u>	5
<u>Aspergillus ustus</u>	5
<u>Emericellopsis synnemata</u>	5
<u>Gliomastix convoluta</u>	5
<u>Penicillium chrysogenum</u>	5
<u>Phialophora</u> sp.	5
<u>Phialophora richardsiae</u>	5
<u>Rhizopus</u> sp. (parasitized)	5
<u>Rhodotorula texensis</u>	5
<u>Stachybotrys atra</u>	5
<u>Achlya klebsiana</u>	5
<u>Cryptococcus diffluens</u>	4
<u>Dictyuchus</u> sp.	4
<u>Graphium</u> spp.	4
<u>Nigrospora sphaerica</u>	4
<u>Paecilomyces elegans</u>	4
<u>Penicillium oxalicum</u>	4
<u>Penicillium sclerotiorum</u>	4
<u>Rhizopus nigricans</u>	4
<u>Scopulariopsis</u> spp.	4
<u>Sepedonium</u> sp.	4
<u>Stemphylium</u> sp.	4
<u>Zygorhynchus moelleri</u>	4
<u>Achlya debaryana</u>	4
<u>Achlya flagellata</u>	3
<u>Allomyces arbuscula</u>	3

<u>Arthrobotrys</u> sp.	3
<u>Candida lusitanica</u>	3
<u>Coniothyrium</u> sp.	3
<u>Fusidium viride</u>	3
<u>Hansenula ellipsoideospora</u>	3
<u>Memnoniella echinata</u>	3
<u>Penicillium janthinellum</u>	3
<u>Penicillium levitum</u>	3
<u>Leptographium</u> spp.	3
<u>Achlya americana</u>	2
<u>Allomyces X javanicus</u>	2
<u>Aspergillus luchuensis</u>	2
<u>Aspergillus nidulans</u>	2
<u>Aspergillus wentii</u>	2
<u>Botrytis cinerea</u>	2
<u>Coccospora</u> sp.	2
<u>Cunninghamella echinulata</u>	2
<u>Fusarium equiseti</u>	2
<u>Fusarium poae</u>	2
<u>Fusarium sambucinum</u>	2
<u>Gongronella butleri</u>	2
<u>Helminthosporium</u> sp.	2
<u>Humicola grisea</u>	2
<u>Mucor foenicola</u>	2
<u>Penicillium frequentans</u>	2
<u>Penicillium ochrochloron</u>	2
<u>Penicillium spinulosum</u>	2
<u>Penicillium thomii</u>	2
<u>Pichia fermentans</u>	2
<u>Rhizopus arrhizus</u>	2
<u>Rhodotorula flava</u>	2
<u>Rhodotorula minuta</u>	2
<u>Saccharomyces montanus</u>	2
<u>Sphaeropsis</u> sp.	2
<u>Spicaria</u> sp.	2
<u>Stilbella bulbicola</u>	2
<u>Syncephalastrum racemosum</u>	2
<u>Torulopsis aeria</u>	2
<u>Trichosporon capitatum</u>	2
<u>Trichosporon margaritifera</u>	2
<u>Trichosporon sericeum</u>	2
<u>Verticillium lateritium</u>	2

The following species were observed only once in this series of samples:

Allomyces macrogynus, Aphanomyces scaber, Ascochyta sp., Aspergillus fischeri, Candida mycoderma, C. ravautii, C. ?zeylanoides, Chaetomium globosum, Cunninghamella bainieri, Cylindrosporium sp., Dendryphion sp., Diplocladium sp., Diplodia zeae, Doratomyces stemonitis, Fusarium roseum, Gliocladium penicillatum, Helicomyces lilliputicus, Hymenula sp., Isaria farinosa, Keratinomyces ajelloi, Monotospora sp., Mortierella sp., Mucor plumbeus, Penicillium adametzii, P. aurantiacum, P. brefeldianum, P. cyclopium var. echinulatum, P. decumbens, P. implicatum, P. javanicum, P. multicolor, P. nigricans, P. pusillum, P. turbatum, P. urticae, P. varians, Periconia sp., Phialophora lagerbergii, Phoma herbarum, Pyrenochaeta sp., Rhizophidium sp., Rhodotorula macerans, R. pennaeus, Saccharomyces carlsbergensis, S. cerevisiae var. tetrasporus, Saprolegnia ferax, Sporobolomyces roseus, Sporotrichum sp., Stysanus sp., Thysanophora penicilloides, Torula herbarum, Torulopsis colliculosa, T. ernobii, Trichocladium asperum, Verticillium sp.

The data in Table 53 are based on colony counts determined for the year during which the waste stabilization ponds were fully operative. They are based on only a few more than 100 of the 212 species obtained during the study. These 100 species are only those which appeared on the pour plates. The figures in the body of Table 53 are based on estimated counts per gram of oven-dry sample based on the average number of colonies per plate poured in replicates of 5, corrected for the appropriate dilution used. The figures for Habitat 1 are these estimated values; the remaining values represent ratios of the other habitats to Habitat 1. The average number of colonies obtained on the eight sampling dates, and the average number of species obtained from each habitat are found in the last two columns. Some locations were sampled fewer than 8 times because some had not been put into operation, others were unavailable because of flooding at various times the ponds were visited, and others for less valid reasons. The number of samples obtained from each habitat is found in the second column.

Ten habitat-types can be observed within the set of habitats described in Table 53:

A - Habitat 1 - Undisturbed soil.

B - Habitat 2 - Raw sewage.

C - Habitats 19-20, 33-34 - Soil samples, paired, from areas with
apparently little or no disturbance.

C-1 - Habitats 19-20 - Marsh areas in Pond 5.

C-2 - Habitats 33-34 - Muskrat holes in dikes of Pond 1.

D - Habitats 3, 4, 5, 14 - Sludges from the bottoms of the primary
ponds near the influent pipes.

E - Habitats 6, 7, 8, 15 - Sludges from the bottoms of the primary ponds near the effluent pipes.

F - Habitats 9, 10, 11, 16 - Mid-pond waters.

G - Habitats 12, 13, 39 - Primary pond effluents.

H - Habitats 21, 22, 23, 24 - Scums from the surfaces of the primary ponds.

I - Habitats 17, 18, 40 - Bottom materials (sands) in the rills in Pond 5 carrying effluents from Ponds 1, 2, and 3.

J - Habitats 25, 26, 27, 28* - Samples from Shaker Creek above pond area.

K - Habitats 29, 30, 31, 32* - Samples from Shaker Creek below pond area.

* 25, 29 - Creek water.

26, 30 - Water and sediment in riffles.

27, 31 - Water and sediment in pools.

28, 32 - Wet bank soil.

In general, the data given on the basis of ratios of different habitats to undisturbed soil indicate only a trend. This trend takes the form of a gradual build-up of colonies as sources of pollution are added to a habitat and are retained on that habitat, although in a flowing condition, for increasing periods of time. Exceptions occur in the case of seasonal variations where colony numbers drop off in autumn, but this may also be related to types of pollutants associated especially with laundry wastes.

In submerged situations, where sludges develop on the bottoms of ponds in which water levels are maintained as well as possible, colony counts are comparatively low. This is especially noticeable for sludges in the vicinity of both influent and effluent pipes in Ponds 1, 2, and 3. Based on plate counts, fungus populations in the waters of the ponds were low. In both situations there were relatively large populations of yeasts as indicated by the results of growth from primary isolation using shaken flasks as well as still cultures in several "enrichment" media. Sludges which had developed in Pond 4, and which never completely dried out although the water seeped out of the pond rapidly, were characterized by much larger populations of filamentous fungi. This is also true of the mud in the swampy areas in Pond 5 irrigated by overflow from Ponds 1 and 2; of the sandy beds of the rills through which that water flowed; the soil around the mouths of muskrat dens on the dikes of Pond 1; and the bank soil along Shaker Creek which was always wet but not submerged. The scums on the ponds which held water were highly erratic in their content of fungi. In the two stations on Shaker Creek the pattern of fungus distribution was not regular. Water was cleaner above the kitchen waste outfall than that below which also received barnyard drainage. Creek bottom sediments were richer in fungi in riffles than in pools but no seasonal pattern of occurrence can be deduced from the data partly because each sample could have been collected from a location different enough to bias the results.

Using the methods of ratios, raw sewage, as it was received by the distributor head, had more fungi than undisturbed soil in seven out of the

eight samples tested. It may be noted that if the ratios were based on raw sewage rather than on undisturbed soil, the patterns of fungus distribution noted above would have been obtained but would have been less distinct because of the larger populations in sewage than in soil.

An attempt was made to develop a matrix based on coefficients of similarity between the several stations tested. It can be noted that 18 of the 36 stations were sampled all 8 times, the remainder were sampled between 3 and 7 times. Colony count information could be used for just over half the species recovered. Based on incomplete data, it appeared unwarranted to complete the development of this technique of determining population differences or similarities.

The numbers of colonies observed on all plates, and the number of yeast cultures isolated from liquid culture, constitute an index of occurrence of a species in a sample or a group of samples. The species with the largest observation number among the 212 species obtained from 8 sets of samples in 4 seasons are listed in Table 55. Observation numbers are given for each of the 11 habitat types described above where the species was observed in one or more of the habitats sampled. Columns A and B represent the single habitats which might contribute to the fungal populations. The undisturbed soil sample is used as a comparison and could represent populations added from soil materials embedded in the clothing worn by farm laborers and laundered on the premises. Column B represents the raw sewage but only a small fraction of this material. Each milliliter of sewage tested could have a quite different assemblage of cells so that it is not surprising that, if sewage were the only source of

Table 53. Distribution of most frequently observed fungi (Number of times observed in eight* sets of samples)

Species

	A	B	C	D	E	F	Habitat Types**	H	I	J	K
<i>Mucor bissealis</i> (ss. lat.)	59	1	1	118	110	7	11	54	108	86	77
<i>Geotrichum candidum</i>	22	26	12	53	60	18	19	68	55	50	54
<i>Trichoderma viride</i>	57	2	29	46	35	5	14	47	44	53	62
<i>Candida brusei</i>	7	5	2	64	53	20	13	45	29	36	60
<i>Fusarium oxysporum</i>	9	3	26	25	31	11	6	6	76	33	28
<i>Rhodotorula mucilaginosa</i>	2	13	17	65	39	37	14	27	27	6	1
<i>Rhodotorula glutinis</i>	2	4	12	29	23	32	15	31	14	2	2
<i>Phialophora jeikei</i>	5	6	4	9	18	11	6	17	17	20	19
<i>Candida tropicalis</i>	-	4	-	25	22	6	3	18	14	12	34
<i>Penicillium lilacinum</i>	7	4	6	15	20	17	9	11	13	16	18
<i>Cladosporium cladosporioides</i>	7	3	7	4	15	13	10	3	15	18	8
<i>Candida parapsilosis</i>	2	6	7	3	11	8	3	12	5	28	18
<i>Fusarium aqueductum</i>	1	1	11	11	7	12	3	8	11	13	15
<i>Saccharomyces cerevisiae</i>	-	1	2	6	7	42	27	13	5	-	1
<i>Aureobasidium pullulans</i>	4	1	7	17	15	21	3	5	4	10	5
<i>Penicillium funiculosum</i>	-	2	7	11	14	3	1	13	16	12	17
<i>Gliocladium roseum</i>	9	1	7	8	10	5	2	7	13	11	14
<i>Fusarium acuminatum</i>	-	-	12	2	2	-	2	-	16	-	13
<i>Epicoecium purpurascens</i>	1	-	8	3	6	5	2	4	6	3	7
<i>Aspergillus flavus</i>	1	1	-	3	8	4	2	9	5	6	8
<i>Aspergillus niger</i>	2	-	21	4	1	1	1	5	5	4	3
<i>Alternaria tenuis</i>	-	2	5	6	5	3	-	5	8	5	1
<i>Aspergillus ochraceus</i>	4	-	7	6	1	2	-	3	7	2	5
<i>Candida guilliermondii</i>	1	4	3	7	2	3	-	2	6	2	7
<i>Aspergillus fumigatus</i>	2	1	2	1	4	-	1	4	6	3	11
<i>Torulopsis inconspicua</i>	-	4	-	5	8	-	2	6	7	-	-
<i>Torulopsis glabrata</i>	-	-	-	13	5	3	2	4	5	3	1
<i>Candida curvata</i>	-	19	-	4	3	4	1	1	3	1	-

*8 sets of samples or fewer as indicated in column 2, Table 53.

** Habitat Types include Habitats as described in Table 53, column 3:

A - 1; B - 2; C1 - 19-20; C2 - 33-34; D - 3, 4, 5, 14; E - 6, 7, 8, 15; F - 9, 10, 11, 16; G - 12, 13, 39; H - 21, 22, 23, 24; I - 17, 18, 40; J - 25, 26, 27, 28; K - 29, 30, 31, 32.

inoculum, an occasional species would be missed in sampling but would contribute to populations in the system. Columns D and E represent a build-up of populations in the sludges on the bottoms of the four ponds; F represents the mid-pond waters, while G is the overflow of these waters in the effluent. Column I represents the rills in Pond 5 which carry the effluent to the marshy areas which developed in that pond represented by Column C-1. These marshy areas show that enough holding capacity was present to retain some water especially after vegetation had built up humic materials. Column C-2 shows that muskrats leaving the ponds and returning to their dens deposit by accident a certain amount of spore-containing water in the soil of the dikes to build up a good population of fungi. Column H shows that scums on the surface of the ponds form a habitat for fungus growth. Shaker Creek, represented by Column J for the station above the influence of the ponds and Column K for the station below the potential influence of the ponds, does not necessarily reflect an influence by the ponds either above or below them. Apparently, it brings with it evidence of an additional source of pollution upstream and is influenced to a certain extent by influents from the dormitory kitchen and the dairy barns.

While some yeast species are present in undisturbed soil samples, the larger percentage of these organisms appear to have been introduced to the system through raw sewage. They multiply well in the ponds and are well represented in the mid-pond waters. It is of interest to note that Saccharomyces cerevisiae, the brewer's and baker's yeast, was present

in a number of habitats throughout the system. It was probably introduced through kitchen baking wastes, and although the raw sewage showed only one cell at the time of sampling, a large population developed in the more liquid phases of the treatment process. In the less liquid phases, and after a month or six weeks, the population died out and did not appear again. One observation of this species was made in Shaker Creek water below the area of the ponds indicating that water seepage through the intervening field could have carried a load of cells. The other yeast species in this list are more frequent inhabitants of sewage polluted habitats and it is more difficult to build statements of their passage through the system.

Discussion

The waste stabilization ponds described here form a planned installation designed to treat the sewage and laundry wastes of a small community, in this case a dormitory. Sewage entering the system carries a high load of solids, colloids, and dissolved materials exerting a high biochemical oxygen demand (BOD), and a low amount of dissolved oxygen (DO). During the period that the sewage flows through the ponds, it loses 70-80% of its BOD, regaining, especially during the day in the presence of algae, some of its DO (Horning et al, 1964). The solids, colloids, and dissolved materials in this sewage constitute an organic enrichment of the habitat. These can act on the fungal spores or cells in the soil as well as those already forming part of the sewage.

Fungi, including filamentous molds and yeasts, were abundantly present in the waters, sludges, and soils of this system as demonstrated by a number of isolation techniques at least one of which was semi-quantitative. Data derived from the use of this technique showed that, depending on the season, the fungi increase as the habitat is enriched with organic wastes. This increase is accompanied by a decrease in BOD for these organisms use only preformed organic matter as food materials.

It is assumed that the original fungal population of the pond system was derived from that already present in the soil. Cells, spores and hyphae were added regularly by the sewage, and other spores and cells were added from laundry wastes resulting from daily washing of the farm laborer's uniforms laden with dirt and manure from the farm, barnyard, and barns. In addition, spores and disseminules from the normal air-spores fallout were probably added to these populations.

A population of slightly more than 5,000 cells per gram dry weight in the subsoil forming the bottom of the ponds was present before sewage was added. Following the initial trials this population was built up to about 24,000 viable cells per gram dry weight of soil. Following a quiescent period, in which the bottoms of the ponds were redesigned to hold water more effectively, sewage was regularly added and population studies initiated. In the spring sampling an average of 250,000 cells of fungi per gram dry weight of sludges and liquids were present; in the summer sampling period this increased to 265,000 cells, by autumn this value decreased to 100,000, and by the following March it had built up again to 990,000 cells, based on data presented in Table 53. These cells

Table 56. Numbers of colonies of fungi per gram dry weight of sample $\times 10^3$ *
(Average of 4 habitats in each pond)

			Numbers of Colonies
July 22, 1957, before initiation of treatment			5.3
February 9, 1959, after preliminary treatment test			24.0
April 1960, through March 1961			
Pond 1	4 - 1 + 18 - 60	Spring	356.0
	7-25 + 9-7-60	Summer	526.0
	10-22 + 11-7-60	Autumn	99.0
	2-24 + 3-13-61	Winter	626.0
Pond 2		Spring	196.0
		Summer	123.0
		Autumn	44.0
		Winter	777.0
Pond 3		Spring	208.0
		Summer	154.0
		Autumn	159.0
		Winter	1,570.0

* Supplementary data on biochemical status of pond waters is found in Horning et al. (1964).

represented at the beginning of the study some 40 species; by the end of the study this number had increased to 212. Data in Table III are aligned to be more easily comparable with data presented by Horning et al (1964).

The actual effect of temperature on fungal populations in this series of habitats and in relation to other organisms present was not tested. Mean temperatures for Pond 1 at 6 inches were: spring 12.3°C, summer 25.7°C, autumn 13.1°C, and winter 10.2°C. The lowest temperature recorded occurred on March 10, 1961, at 18 inches in Pond 3: 4.0°C. In the summer 30°C was recorded on three different days at 6 inches in both Ponds 1 and 3 (Horning et al, 1964). On the basis of available data, there appears to be no relationship between temperature and population levels represented by numbers of colonies or species of fungi.

Aside from indicating that potentially initial populations of the area represented by undisturbed soil and raw sewage are different, and that populations found in the two stations on Shaker Creek are different from each other and from populations in the main stabilization pond area, similarity coefficient analyses yielded no important information. This is partly because of insufficient sampling at some sites, partly because of the nature of the basic food materials (sewage) and the nature of the introduced populations which accompanied the sewage.

Although fungal populations were obviously active in all parts of the system, it is not obvious in what life form these organisms occurred. Yeasts were probably present as colonies of separate cells, just as were many of the bacteria. The filamentous fungi, including most of the species

occurring in semiquantitative studies, were probably present as filaments of fungus tissue. In a mass of sludge, the filament can grow onto a second food source as a first one becomes exhausted. While bacteria and probably yeasts are present in the waters and possibly in the sludges in large numbers, there is no indication of the relative amount of food supplies each type of organism in each habitat is able to remove from that habitat.

Cooke et al (1956) showed that at the pH levels found in the ponds fungi alone utilize weak nutrients (300 ppm glucose - glutamic acid) more slowly than do sewage seed, or a mixture of fungal cells and sewage seed (which includes fungal cells), as measured by the decrease of BOD in a 10-day laboratory culture. Since fungal growth occurred at least in both types of culture to which fungi were added, problems of competitive advantage were probably overcome by the nature of the groups of organisms involved. In the waste-stabilization ponds, except for November when adverse conditions of undetermined nature (possibly related to wastes added to the ponds) reduced the fungal populations, the numbers of viable cells recovered indicate a favorable habitat for fungi. On this basis, it appears that fungi can be counted among those saprobic organisms that play a part in the degradation and thus the removal of organic wastes from the waters of waste stabilization ponds.

Conclusion

The development of a population unit depends on either the appearance of new ground, or the disappearance of an existing population. In this

pond system, an existing population was partially eliminated in the clearing of ground which essentially resulted in the appearance of new ground. As the ponds began to function, the development of a biotic association resulted from the interaction of organisms, available population, and environmental selection. The environment selected from all available immigrants those species that constituted the populations found during the study. This population varied continuously in time and space. Each organism seized and used the particular time-period during which the environment was in a condition suitable for it. These factors were reflected by the changing patterns of the population members in the pond system and its environs during the single year of observation. Within the ponds the preformed organic matter comprising the bulk of the organic pollutants in the sewage formed the basis for selection of the species coming to reside in the ponds; as the nature of these pollutants changed, populations declined or increased throughout the year. Factors of regular or irregular environmental changes, together with the facts that species of fungi have reproductive capacities beyond their needs, and that fungi are highly plastic in their capacity to respond to habitat variables, were demonstrated by the fungus populations found in the Lebanon waste stabilization ponds. As the use of waste stabilization ponds for sewage treatment becomes more common, as their design characteristics become better understood, and as all the elements of a pond system are put to their maximum use, the fungi, as well as other organisms in the system, will be used to increasing advantage in the treatment of organic wastes.

B. Species of Fusarium isolated from the Lebanon ponds

Among the fungi isolated from the Lebanon waste stabilization ponds are species which are known to cause disease in wild and crop plants. Since no tests have been made of the strains isolated at Lebanon in relation to their pathogenicity or lack of it, they can only be assigned to known species as potential pathogens. Within this category can be placed some of the 9 species of Fusarium which were recovered. These are listed in Table 57 together with a code letter to be used in Table 58, and the number of isolates made of each species. It will be noted that F. oxysporum, F. aquaeductuum, and F. acuminatum were the commoner species in that order.

Various species of Fusarium respond differently to the different types of habitat in which they are found. Species are present in all sludges and waters in the system, as shown by the first section of Table 58, but are more abundant where the ponds have dried out as at stations 14 and 15 of Pond 4; in soils, although wet and swampy, larger numbers of isolations were made as indicated by the second part of Table 58. In the adjacent stream, species were found wherever samples were taken but were more abundant in bank soil than in riffles, pools or waters that had floating vegetation to which colonies could attach.

Species of Fusarium react differently to the various methods of isolation. For instance, F. aquaeductuum was rarely obtained when liquid cultures were used, as in the shaker isolation technique for yeasts and in the still culture enrichment media. Possibly this is a reaction to

Table 57. Fusarium species isolated from Lebanon Ponds.

Code letter	Section and Species	Number of isolates
	Eupinnotes	
A	<u>F. aquaeductuum</u> (Radlk. & Rabh. pr.p.) Lagh	102
	Sporotrichiella	
B	<u>F. poae</u> (Pk.) Wr.	2
	Gibbosum	
C	<u>F. acuminatum</u> (Ell. & Ev.) Wr.	61
D	<u>F. equiseti</u> (Cda.) Sacc.	2
	Discolor	
E	<u>F. graminearum</u> Schwabe	18
F	<u>F. sambucinum</u> Fuckel	3
	Liseola	
G	<u>F. moniliiforme</u> Sheld.	8
	Elegans	
H	<u>F. oxysporum</u> Schl. em. Snyder. & Hans.	216
	Martiella	
I	<u>F. solani</u> (Mart.) App. & Wr. em. Snyder. & Hans.	21
	Total Isolates	433

Table 58. Species of *Eugarium* in waste stabilization pond habitats.

Habitat*	7-22-57	2-13-59	4-1-60	4-18-60	7-25-50	9-7-60	10-22-60	11-7-60	2-24-60	3-13-60	Total Obs- erva- tions	Total spec- ies
2	HI**			A	I	A	AH	H		H	4	3
3	A			A	I		AH			AH	9	3
4				A	I		AH	H		H	2	2
5					I		AH	H	H	H	6	3
14				AH			AH	ACH	A	ACH	8	4
6	H						AH	H		H	7	3
7							AH	H		H	2	1
8		A			I		AH	ACH	H	H	4	2
15		A		A		A	H			ACH	10	5
9							AH			H	5	2
10				A	A	A	A	A	G	H	3	2
11				A	EI		A			AH	6	3
16				A	C		A			ACH	6	5
12							A			H	3	3
13							SHI			H	1	1
39							H	H	EH	AH	8	5
21			H			A	A				4	2
22	DHI					A	A				4	4
23				A	A	A	A			H	6	4
24					A	A	A				1	1
1	HI		EH	AH	I		A	H		EH	9	4
17			CH	ACH		I	H	AGHI	H	AH	13	5
18		A		ACH	E	AE	AH	ACH		ACH	15	5
19	AF			AG	BEH		AH	ACH		ACH	18	7
20				A	I	AH	ACH	CH	A	H	9	4
40							ACH	ACH		CH	11	4
33			H	A	A		ACH	ACH	EH	ACH	12	5
25						A	A			AH	3	2
26					AI	A	ACH	AH			8	4
27			H	D			ACH	ACH			6	3
28			H	AH	A		ACH	ACH	A		12	5
29				A	A	A	ACH	ACH			5	2
30				A			ACH	ACH			7	3
31			F	G			ACH	ACH			7	3
32			CH	ACH	A	A	ACH	ACH			13	4
35*				A	H		ACH	ACH			2	2
36*							ACH				3	3
37*							ACH			ACH	3	3
38*							ACH				3	2
Total Stations 6	3	11	11	18	19	14	29	21	9	26	39	
per date yielding species of <i>Eugarium</i> .												

* See Table 53. ** See Table 57.

crowding for this is a relatively slow-growing species which does very well on dilution plates as Table 59 indicates. On the dilution plates this species was found at 36 of the 39 sampling points in spite of the fact that rose bengal was used in the medium. However, until enrichment media in liquid culture were tried, F. oxysporum was found infrequently (Table 60). In the liquid culture techniques it was found that this fungus was very common throughout the series of habitats sampled. Other species of Fusarium gave more erratic patterns of distribution with only an occasional isolate or no specific distribution pattern.

On the basis of the isolates of Fusarium observed during this study, it appears that several species potentially pathogenic to plants may occur in the ponds at Lebanon. However, since only a few strains of the more important pathogenic species are known to cause diseases of plants, it is possible that no phytopathological significance can be attached to the occurrence of these fungi in waste stabilization ponds.

On the other hand, it seems quite likely that should a pathogenic strain reach a series of habitats such as the one described here, it could adapt to the enrichments resulting from the addition of sewage and develop rapidly; the area then could become a potential hazard to the farm community. Effluents from a pond system can readily carry spores from place to place, and in dryer areas effluents from such ponds may be developed as irrigation supplements. Under certain conditions sludges may be removed and applied to soils as fertilizer supplements. In either or both cases a heavier than normal spore load is applied to the soil, the effects of which can only be anticipated.

Table 59. Occurrence of *Fusarium aqueductum*.

Habitat Number*	7-22-57	2-13-59	4-1-60	4-18-60	7-25-60	9-7-60	10-22-60	11-7-60	2-24-61	3-13-61
1							R			
2	R**			R		R, 20	R			R
3				R			R			
4							R			
5							R			
6							R			
7							R			
8							R			
9							R			
10							R			
11							R			
12							R			
13							R			
14							R			
15							R			
16							R			
17							R			
18							R			
19							R			
20							R			
21							R			
22							R			
23							R			
24							R			
25							R			
26							R			
27							R			
28							R			
29							R			
30							R			
31							R			
32							R			
33							R			
34							R			
35							R			
36							R			
37							R			
38							R			
39							R			
40							R			

* See Table 53.

** See Table 60.

Table 7. Occurrence of *Fusarium oxysporum*.

Habitat Number*	7-22-57	2-13-59	4-1-60	4-18-60	7-25-60	9-7-60	10-22-60	11-7-60	2-24-61	3-13-61
1	R							CE		ACH
2	R							RI		R
3							A			R
4							DEGJ			RAC
5							APG	D	R	R
6	R							J		RJ
7								CH		RG
8							IJ			R
9							ACDEJ			R
10										R
11										R
12										R
13										R
14										R
15										R
16										R
17										R
18										R
19										R
20										R
21										R
22										R
23										R
24										R
25										R
26										R
27										R
28										R
29										R
30										R
31										R
32										R
33										R
34										R
35										R
36										R
37										R
38										R
39										R
40										R

* See Table 53.

Letter Symbols: R - dextrose-neopeptone-rose bengal-aureomycin agar; 1, 20 - YCB-1% or 20% dextrose broth shaken 50 hours; A - YCB - (H₂)₂SO₄; C - YCB-KNO₃; D - YNB-dextrose; E - YNB-lactose; F - YNB-galactose; G - YNB-sucrose; H - YNB-sucrose; I - YNB-ethanol; J - YNB; K - YCB + YNB.

C. Yeasts in Waste Stabilization Ponds

Isolation Techniques

Samples from the habitats described above were returned to the laboratory and were inoculated on the same day. Agar for plating had been prepared in advance and was held in the liquid state in a water bath at 55°C. Dilution water was ready for use. Other media were ready for inoculation. Fifteen ml of the sample were added to 150 ml of distilled water in 250 ml Erlenmeyer flasks, placed on a rotary shaker, and shaken for 30 minutes to blend the sample as much as possible and to break up spore and cell clumps. The flasks were then removed from the shaker, and 5 ml of this material added to 45 ml of distilled water, when dilutions greater than 1:10 were desired; this operation was repeated for dilutions of approximately 1:1000 and 1:10,000.

In sample plating, 1 ml of the final dilution, based on earlier experience with the type of sample, was added to each of five tubes, each containing 10 ml of neopeptone-dextrose-rose bengal agar to which had been added 0.05 ml of a 1-g/150-ml solution of aureomycin HCl. This mixture was then poured in 9-cm Petri dishes, allowed to harden, and incubated 7 days at room conditions of temperature and light. At the end of this time, colonies of fungi appearing on the plates were counted and their genus or species sight identified. Yeast colonies were selected, as were other unidentified fungi, for future study. Yeast colonies observed in this way were the only ones for which semi-quantitative data were obtained.

A second method for isolation of yeasts was used for all samples. A solution containing 6.7 g per liter of Wickerham's YNB medium (Difco dehydrated, 1953) was prepared. To half of this solution 1% glucose was added, to the other half 20%. The YNB and glucose solutions were sterilized by membrane filtration. Reproduced with permission from *Protoplasma* 57:163-187, 1963.

These were dispensed at 50 ml per flask in 250-ml Erlenmeyer flasks, and 1 ml of the 1:10 dilution of each sample was added to each of these media. The flasks were then placed on the rotary shaker, shaken for 64 hours, and removed, and the growth allowed to settle. In general, the yeasts settled to the bottom, the bacteria remained in suspension, and the molds remained at the surface or in suspension. Plates of yeast extract-malt extract-glucose agar had been prepared, and on to these plates in duplicate or triplicate were streaked loopfuls of yeast cells that had settled to the bottom of the incubation flasks. These plates were incubated for 2-3 days, and from the resulting growth, yeast colonies were picked, or where colonies were not discrete, masses of yeast cells were streaked on to Diamalt agar plates. Through additional streaking or dilution techniques the colonies were finally secured in pure culture and then identified by the Delft scheme supplemented by use of the ^{14} C-carbon sources for assimilation patterns, as suggested by Wickerham and Burton (1948).

A method of isolating yeast colonies by enrichment culture was suggested as a means of obtaining a more representative series of yeast cultures. Rather than the various types of natural products in the media, it was decided that a reproducible set of media would serve our purpose. The selected media followed the Delft identification scheme for carbon and nitrogen utilization tests (Lodder and Kreger-van Rij, 1952). Thus three nitrogen sources, $(\text{NH}_4)_2\text{SO}_4$, NaNO_2 , and KNO_3 , were incorporated in YCB broth; six-carbon sources, glucose, lactose, galactose, maltose, sucrose, and ethanol, were used in YNB broth; YNB and YCB media were blended in one broth; and VFYB (vitamin-free yeast base) was included in one broth. These several broths were prepared in 20-ml lots in 20 by 150 mm culture tubes capped with aluminum caps. Sterilization was

accomplished by passing the broth through membrane filters. One ml of the 1:10 dilution of each sample was inoculated into each medium, 0.1 ml of the aureomycin solution described above was added to each tube to prevent undue bacterial contamination, and the whole lot incubated for at least 7 days at room conditions of temperature and light. No attempt was made to prevent the growth of filamentous fungi, which appeared in almost all tubes.

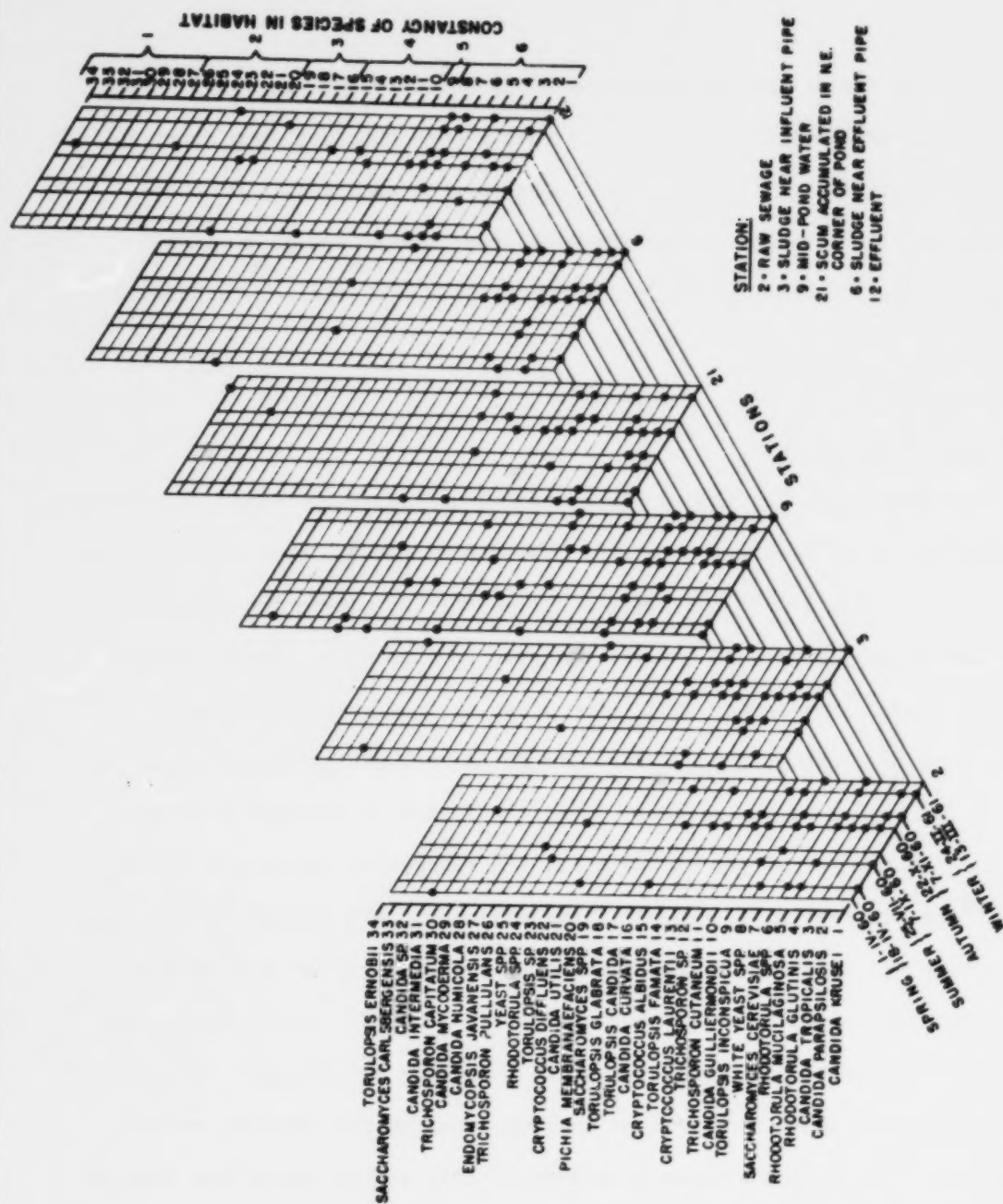
Two types of agar were prepared on which to plate the resulting growth. Instead of glucose, except in one case, neopeptone-rose bengal agar was prepared containing one-half the nitrogen or carbon source used in the corresponding incubation tube. To this was added aureomycin in half the amount used in the earlier plating technique. Two plates of Diamalt agar were prepared for each sample. From each of the primary incubation tubes three plates were poured. In the first set of samples in which this technique was used, the whole mass of material in the tube was divided between one rose bengal agar and two Diamalt agar plants. In later experiments, 1 ml of suspension was added to each plate. Plating was carried out by adding the suspensions to empty plastic Petri dishes, then pouring in the melted agar, and mixing. Mold growth that appeared as contamination on the sample was recorded, giving information in addition to that obtained from the two techniques described above. Hemp seeds as bait for aquatic fungi, and, in one case, pieces of wool cloth as bait for keratinophilic molds, were also tested. From those plates that were not too heavily overgrown with molds, yeast colonies were isolated and retained for identification. In some cases molds such as *Mucors* and *Rhizopus* that completely smothered yeast colonies were burned away with red-hot nichrome wire needles. Such colonies had to be purified by one or more transfers to

Diamalt agar to remove the contaminating molds and sort out the mixed yeasts. In these ways more than 1800 cultures of yeasts were obtained from about 280 samples of water, sewage, sludge, soils, and related materials. Of those 1800 cultures, 1698 have been identified or brought near enough to final identification to be used in the following analysis of isolation techniques and populations.

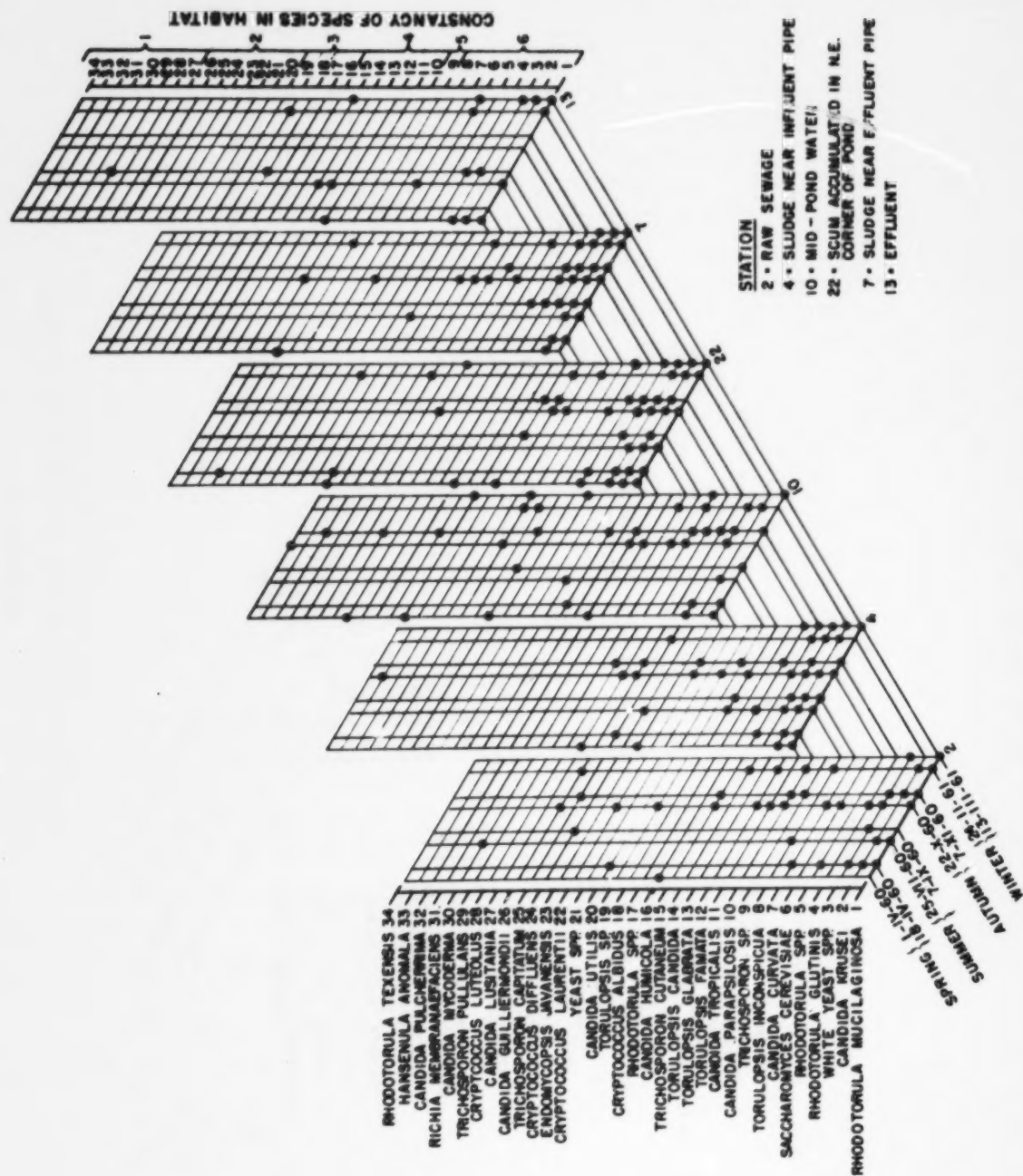
Results

Populations of Yeasts

The three principal techniques described were used to obtain 54 species or species-groups of yeasts from the 40 habitats sampled. These habitats have been aligned in relation to the pattern of sewage flow within the system, since this is the primary source of habitat change. For instance, Fig.24 presents a set of histograms showing the occurrence of yeast species within Pond No. 1, starting with raw sewage, progressing through the sludges accumulating in the bottom of the pond near the influent pipe, the liquid in the middle of the pond, the scum on the surface of the pond, the sludges on the bottom of the pond near the effluent pipe, and the final effluent. All samples include some pond water, since this cannot be satisfactorily excluded from grab samples. Figs.25 and 26 give similar information for Ponds 2 and 3. Fig.27 shows the distribution of yeasts in Pond 4 from which there was no effluent. Mid-pond waters and surface scums were present in Pond 4 during few sampling periods, and therefore, the bottom sludges were sampled dry; dry or nearly dry organic matter and soil were scooped from the pond bottom. Fig.28 shows the distribution of yeasts in undisturbed soil, soils on the dikes around Pond 1, and

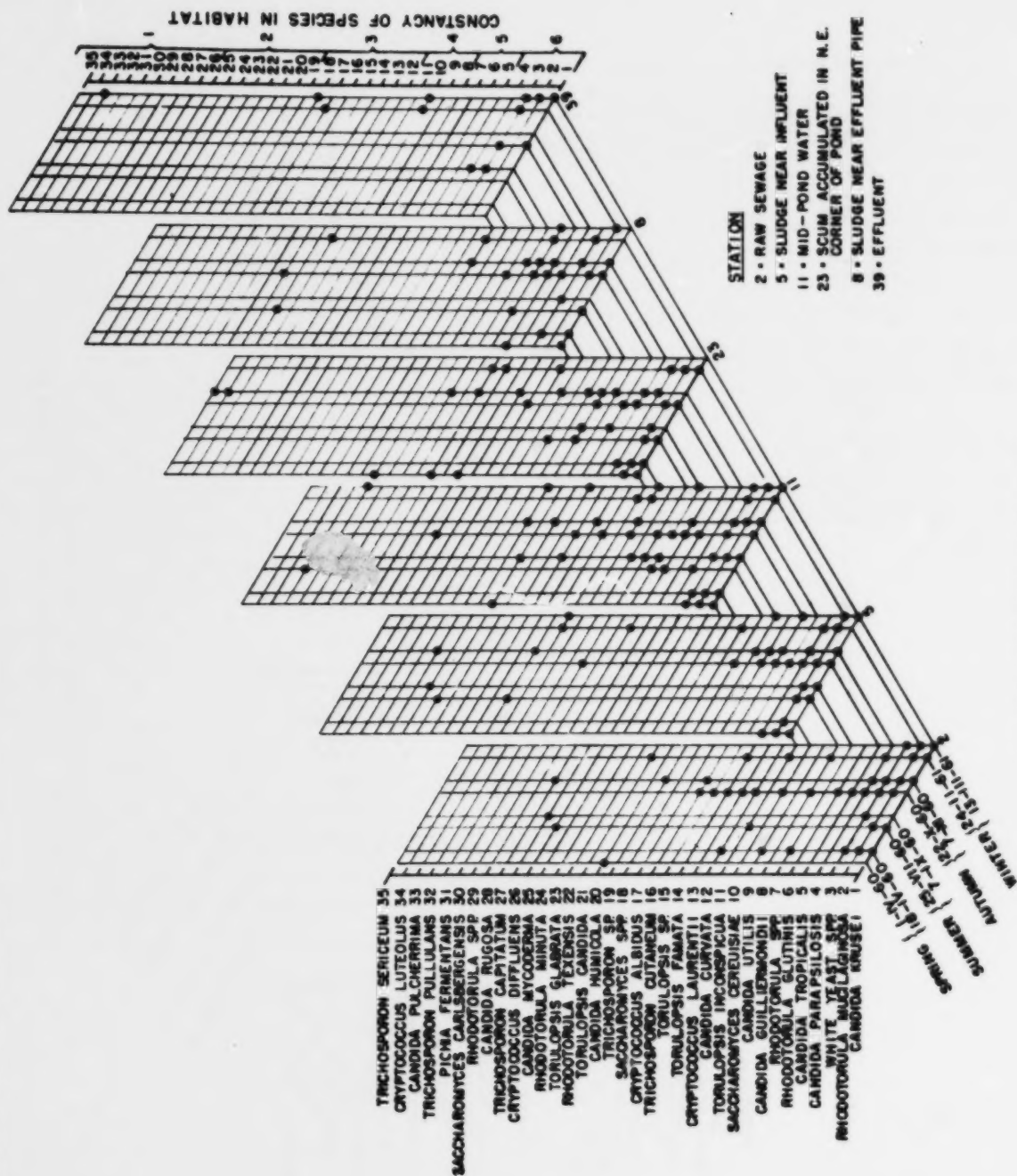


YEAST SPECIES IN POND 1
LEBANON WASTE STABILIZATION POND SYSTEM
FIGURE 24



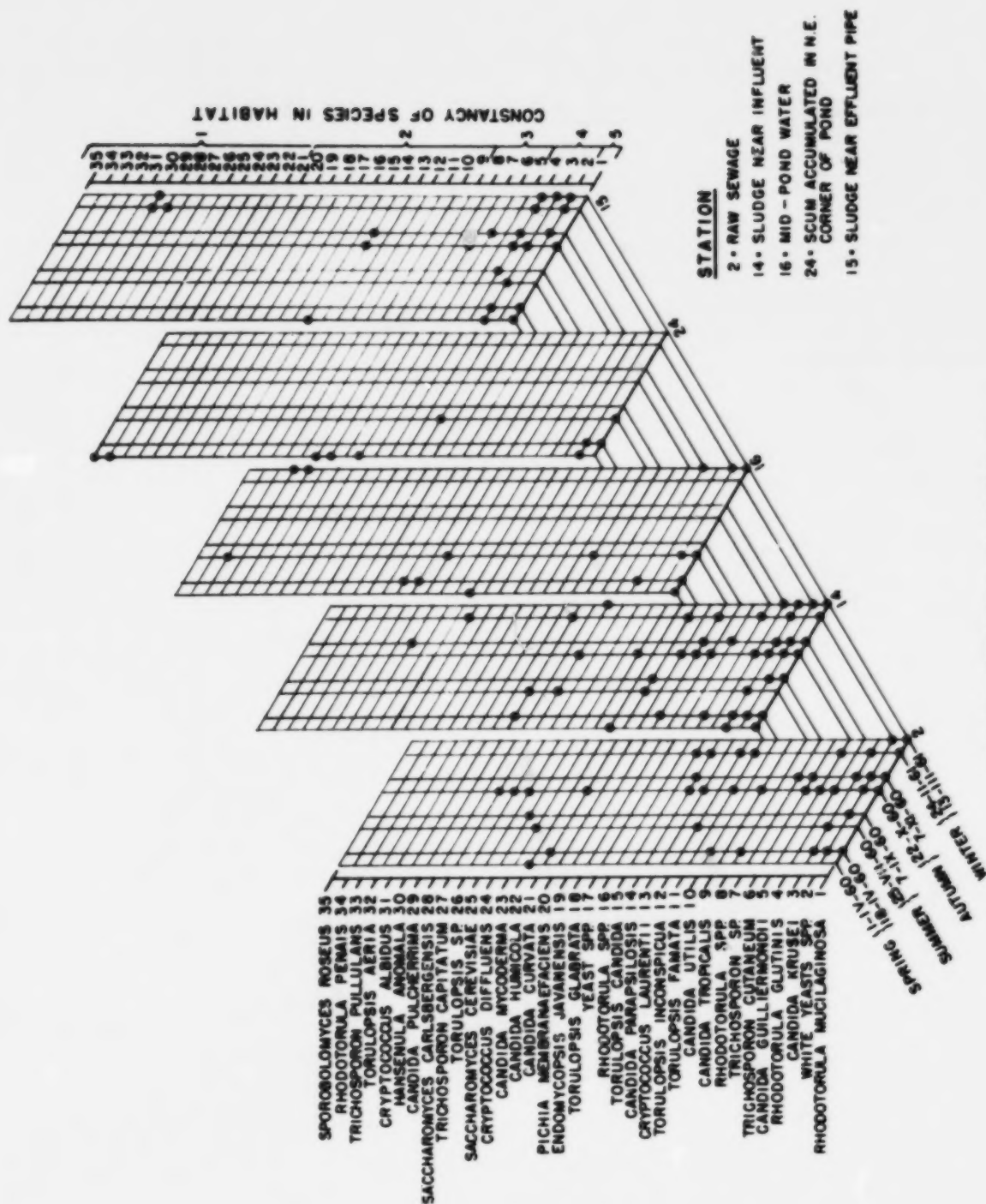
YEAST SPECIES IN POND 2
LEBANON WASTE STABILIZATION POND SYSTEM

FIGURE 25

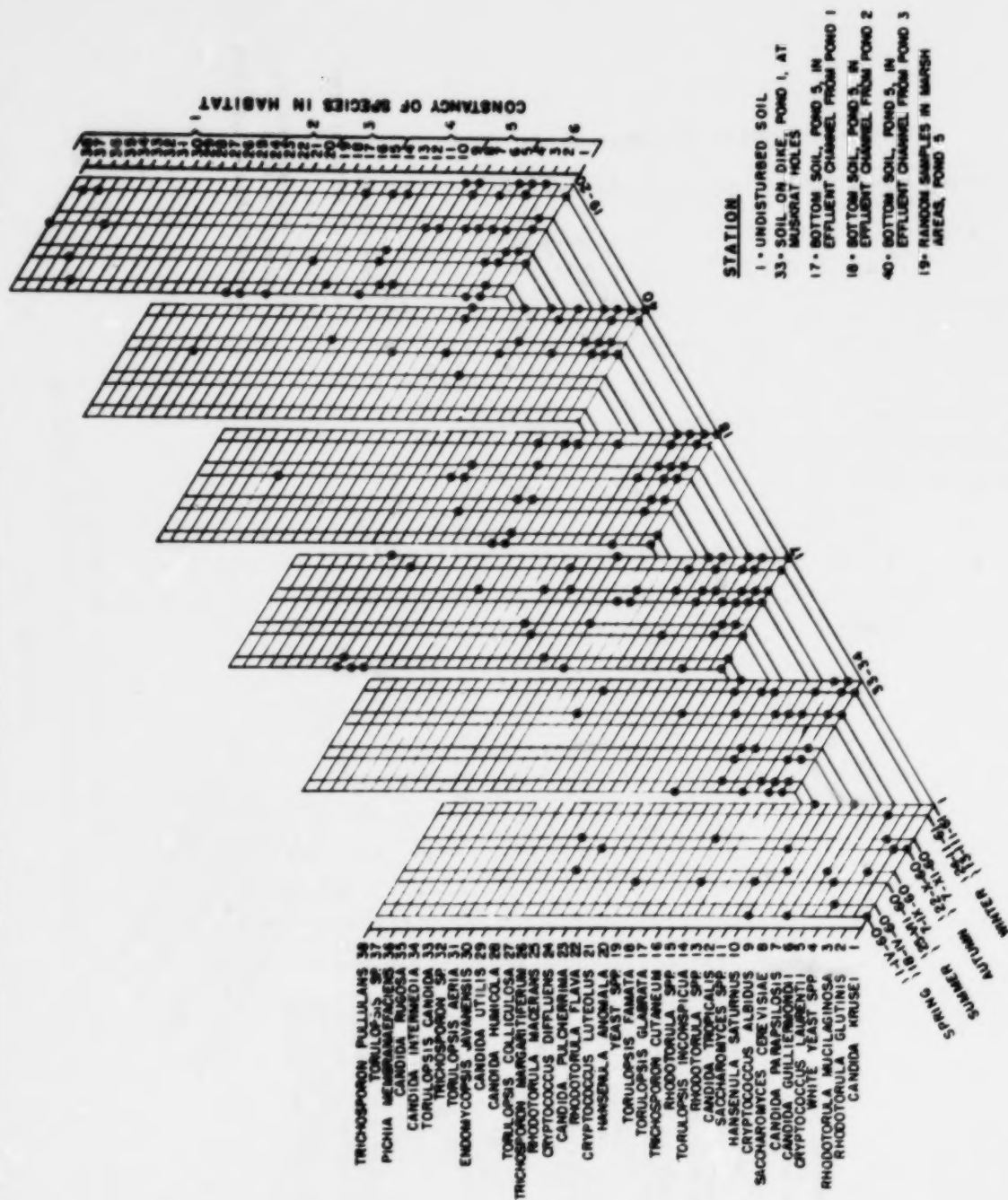


YEAST SPECIES IN POND 3
LEBANON WASTE STABILIZATION POND SYSTEM

FIGURE 26



YEAST SPECIES IN POND 4
LEBANON WASTE STABILIZATION POND SYSTEM
FIGURE 27



YEAST SPECIES IN BOTTOM OF POND 5 AND OTHER SOILS

FIGURE 28

soils affected by the discharges from Ponds 1, 2, and 3 in Pond 5. The populations present in Shaker Creek are given in the two sets of histograms in Fig. 29. These histograms illustrate the four habitats sampled at the upstream station and those sampled at the downstream station. Fig. 30 summarizes these 6 sets of habitats without regard to the individual habitat within each set.

As is true of other types of organisms in other types of habitats, a few species are found in almost every location on almost every sampling date. Thus, of the first 10 species on the summary histogram, not including categories with more than one unidentified species, six were found in Pond 1, five in each of Ponds 2 and 3, four in Pond 4, six in the different soils sampled, and five in Shaker Creek. These first four, five, or six species may not always have been isolated on the same date and they may not always have been isolated from the same types of habitat; however, at least four of them are consistently present throughout this group of samples. It may be said, on this basis, that Candida krusei, C. parapsilosis, C. tropicalis, Cryptococcus laurentii, Rhodotorula glutinis and R. mucilaginosa, within this particular set of habitats, are characteristic of situations that have been enriched by the organic matter of domestic sewage.

Ecological Considerations

In the literature of ecology the quantitative description of populations is best demonstrated for populations of higher plants wherein the numbers of individuals of herbs, shrubs, and trees can be obtained by direct counts. Constancy, fidelity, dominance, and other characteristics of one or more associations in a population can be obtained directly. As the size of individuals decreases, it becomes less and less easy to demonstrate these features of a population.

Below the level of mosses and liverworts, some of whose individuals are difficult to distinguish in a close association, it is not always practical to evaluate populations quantitatively.

Dr. Höfler (1955) and his students have pioneered in the work of describing associations of soil organisms represented by the larger fungi whose fruit bodies are usually referred to as mushrooms. Different techniques of quantitatively determining populations of fungi have been suggested by Wilkins (Cooke 1953) and his students, Parker-Rhodes (Cooke 1953), the writer (Cooke 1955), and others (Bohus 1960).

Of the smaller organisms, the algae (Fetzmann 1956) have been studied intensively by students of Dr. Höfler and useful techniques have been suggested for describing quantitatively the populations of diatoms, desmids, filamentous algae, and similar organisms found in lakes, ponds, and streams. For this type of study the organism must be large enough to be observed and studied under lower powers of the microscope. These organisms are usually pigmented or have specially marked shells that render them easily observable among other populations in gross mounts of water from the habitat whose populations are being described.

At still lower levels from the point of view of size, are the bacteria, which, except for those that produce filaments or iron incrustations, can not be observed in a raw water sample from any habitat without elaborate staining or culture techniques; and the fungi, whose spores are rarely noted in spot checks of plankton or benthos samples and whose mycelia in such samples are impossible to identify because all mycelia in aquatic habitats are similar in morphology.

To adequately demonstrate the populations of bacteria and fungi, including yeasts (which have rarely been reported in plankton analysis work), mixed or pure culture techniques are among the few methods available. As a means of describing quantitatively the populations of yeasts in a habitat, no previously described model has been found either in studies of bacteria or filamentous fungi. Estimates of total populations or of fractions of populations capable of one physiological process or another, or of causing one disease or another have been made. No study has been found, however, in which the members of a total population of microorganisms, whether bacteria, fungi, or protozoans or combinations of these, were related to each other, as are the members of an association such as a beech forest.

In one isolation technique used in the survey reported here, the yeasts were listed in a semiquantitative manner. If five colonies were found on a plate poured from a dilution of 1:10,000, this would indicate that in the original sample there might have been 50,000 cells of this yeast per ml. But, if from a 1:10 dilution of this same sample only five of the other 13 media used yielded this species, would this mean that only 50 cells were present in the rest of the sample? If no yeasts appeared on the dilution plates from another sample, even though this species grew in 12 of the other 13 media used, how could its relative abundance be evaluated? Obviously, another isolation technique must be tried, one with a different approach to quantitative methods.

In his recent volume on soil microbiology, Alexander (1961) suggested, in work on plant disease fungi, that there is a quantitative technique available for their study. This is a modification of the MPN technique developed

for work in water bacteriology and will be adapted to future work with yeast populations.

Total Species

In a study of mushrooms in a peat bog in Denmark, M. Lange (1948) found that as a result of one observation, corresponding to one collecting trip, he could anticipate finding 50 percent of the species present. After four sets of observations 80 percent of the species would be reported, and after eight observations 85-97 percent of the species would be reported, depending on the season and whether all species fruited within one series of observation periods. Thus, in collecting fruit bodies of larger fungi, as well as in other work with biological materials, the law of diminishing returns applies.

Cumulative summaries of the yeast species found in nine different habitat types in the Lebanon ponds are presented in Tables 61 and 62. The data are arranged in two ways. In Table 61 the nine habitat types are presented, and for each collection date, the total number of species found to that time and the percent of the total number found are listed. In this summary an average percent of occurrence is given for each date. Since the ponds have been abandoned, the habitats can be considered eliminated and the 54 categories listed can be considered the complete population, except for revisionary data resulting from completion of identification of four categories of unidentified cultures. In Table 62 the total number of species found in each of the nine habitat types is listed in a cumulative manner, with the percent of the actual total species found given. The theoretical total of 54 should prove to be 90 percent of the actual total, according to the observations of Dr. Lange.

Table 61. Cumulative summary of yeast species found in 9 habitat types (Numbers and percent by date).

Habitat Types	Collection Dates							
	1.IV.60 No. %	18.IV.60 No. %	25.VII.60 No. %	7.IX.60 No. %	22.X.60 No. %	7.XI.60 No. %	24.II.61 No. %	13.III.61 No. %
1. Undisturbed Soil	3 18.8	4 25.0	8 50.0	12 75.0	14 87.5	16 100.0	16 100.0	16 100.0
2. Raw Sewage	1 4.8	7 33.3	9 42.9	10 47.6	18 85.7	20 95.2	21 100.0	21 100.0
3. Pond 1	11 35.5	13 41.9	15 48.4	19 61.3	23 74.2	28 90.3	30 96.8	31 100.0
4. Pond 2	13 40.6	15 46.9	18 56.3	20 62.5	26 81.3	30 93.8	32 100.0	32 100.0
5. Pond 3	9 28.1	9 28.1	15 46.9	17 53.1	22 68.8	27 84.4	29 90.6	32 100.0
6. Pond 4	10 34.5	14 48.3	18 62.1	18 62.1	22 75.9	24 82.8	27 93.1	29 100.0
7. Modified Soils	15 41.7	19 52.8	21 58.3	23 63.9	28 77.8	31 86.1	35 97.2	36 100.0
8. Shaker Creek Upstream	9 37.5	9 37.5	11 45.8	15 62.5	20 83.3	22 91.7	24 100.0	24 100.0
9. Shaker Creek Downstream	8 36.4	11 50.0	15 68.2	15 68.2	16 72.7	17 77.3	20 90.0	22 100.0
Average % occurrence	30.9	40.4	53.2	61.8	78.6	89.1	96.5	100.0

Table 62. Cumulative number and percent of total species found in each habitat type.

	Habitat Type (Table 61.)								
	1	2	3	4	5	6	7	8	9
Number species found	16	29	38	40	47	50	52	54	54
% total to date (54)	29.6	53.7	70.4	74.1	87.0	92.6	96.3	100.0	100.0
% potential total (60)	26.7	48.3	63.3	66.7	78.3	83.3	86.7	90.0	90.0

Constancy

In Figs. 24 through 30 the species numbers in the column on the left of each figure are bracketed. The brackets include those species found in all the habitats presented in each figure, or in only some or one of these habitats. It will be noted that the species listed (Table 63) as appearing most frequently in response to the changing organic matter composition of the habitat are those bracketed among the species appearing in all habitats on one or more sampling dates.

Fidelity

If the percentages of species of organisms of one habitat or association that are also found in each of the other habitats or associations in a study area are plotted in a series of histograms, the fidelity of the species found in the other habitats to that group of species can be judged. Series of such histograms are illustrated in Figs. 31 to 37. For this analysis the same groups of habitat types are used as those used for describing the populations of yeasts in Figs. 24 through 27.

A fairly good correlation between all habitat types affected by sewage is noted in this analysis. Populations are decreased as water seeps away from a pond as shown in column 6 for Pond 2 (Fig. 32) and column 6 for Pond 3 (Fig. 33). Shaker Creek populations (Fig. 13) show a lower correlation with other habitats sampled probably because fewer species with true mycelium and even pseudomycelium, and with red pigment, occur in this set of habitats than in the other habitats.

Other Ecological Considerations

Because of the limited population being considered here and because quantitative techniques have not been used to describe more than a small

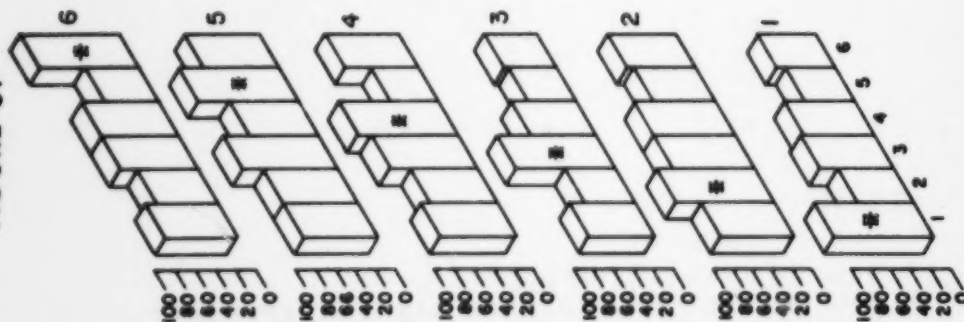
Table 63. Percent of each yeast species in all habitats on all dates in each medium.

Species	R	Media (see Table VII-12)								
		1	20	A	B	C	D	E	F	G
Undetermined Yeasts	43.9	11.9	10.4	1.1	1.9	3.7	1.9	5.6	2.6	3.7
<u>Candida krusei</u>		38.5	22.2	4.2	1.0	2.4	3.8	2.1	2.1	5.6
<u>Rhodotorula mucilaginosa</u>	6.3	24.0	31.7	2.3		1.4	4.1	4.1	1.4	5.9
<u>Rhodotorula glutinis</u>	2.5	18.0	16.8	7.5		1.9	8.1	5.6	5.6	6.2
<u>Candida tropicalis</u>	0.9	33.3	28.2	2.6		2.6	3.4	2.6	6.0	5.1
<u>Saccharomyces cerevisiae</u>	3.0	11.9	9.9	13.9		8.9	10.9	2.0	10.9	8.9
<u>Candida parapsilosis</u>		23.0	33.0	5.0		3.0	1.0	5.0	7.0	6.0
<u>Candida guilliermondii</u>	2.7	21.6	35.1	5.4					5.4	5.4
<u>Torulopsis inconspicua</u>	19.4	13.9	17.7	8.8		2.9	11.8	2.9		5.9
<u>Trichosporon cutaneum</u>	12.5	18.8	9.4	9.4		3.1	6.3	9.4	9.4	
<u>Cryptococcus albidus</u>	23.3	10.0	13.3	16.7		3.3		10.0		10.0
<u>Trichosporon</u> sp.		15.8	5.3	5.3		5.3	10.5	31.6	10.5	5.3
<u>Candida curvata</u>		20.8	16.7	4.2			4.2	29.2		4.2
<u>Cryptococcus laurentii</u>		7.4	25.9				3.7	33.3		3.7
<u>Torulopsis famata</u>		34.5	44.8	3.4				3.4	3.4	
<u>Hansenula saturnus</u>	7.7	38.5	7.7				7.7			7.7
<u>Torulopsis candida</u>	8.3	16.7	41.7	8.3				8.3	8.3	
<u>Candida utilis</u>	7.1	28.6	28.6	14.3				7.1		
<u>Candida pulcherrima</u>	9.1	27.3	45.5						9.1	
<u>Endomycopsis javanensis</u>	9.1	72.7	18.2							
<u>Candida rugosa</u>	12.5	62.5	25.0							
<u>Pichia membranaefaciens</u>	14.3	42.9	14.3							
<u>Rhodotorula texensis</u>	40.0	20.0					20.0	20.0		
<u>Cryptococcus diffluens</u>	75.0									25.0
<u>Rhodotorula minuta</u>	50.0									
<u>Trichosporon capitatum</u>	50.0								50.0	
<u>Torulopsis glabrata</u>		41.2	47.1	5.9		2.9				
<u>Candida intermedia</u>		38.5	53.8							
<u>Hansenula anomala</u>		9.1	27.3	9.1				18.2		9.1
<u>Torulopsis</u> sp.		22.2		11.1		11.1				11.1
<u>Trichosporon pullulans</u>		22.2	33.3					11.1		
<u>Candida humicola</u>		75.0	25.0							
<u>Cryptococcus luteolus</u>		37.5	50.0					12.5		
<u>Candida lusitania</u>		66.7	33.3							
<u>Hansenula ellipsoidospora</u>		33.3	33.3							33.3
<u>Rhodotorula flava</u>		50.0	50.0							
<u>Candida mycoderma</u>	100.0									
<u>Saccharomyces</u> sp.	100.0									
<u>Pichia fermentans</u>			50.0			50.0				
<u>Trichosporon margaritiferum</u>			50.0	50.0						
<u>Rhodotorula macerans</u>			100.0							
<u>Rhodotorula pannaecus</u>			100.0							
<u>Sporobolomyces roseus</u>			100.0							
<u>Torulopsis colliculosa</u>			100.0							
<u>Saccharomyces carlsbergensis</u>				33.3			33.3			
<u>Torulopsis aëria</u>				50.0						
<u>Trichosporon sericeum</u>								100.0		
<u>Torulopsis ernobii</u>									100.0	
<u>Candida brumptii</u>										100.0

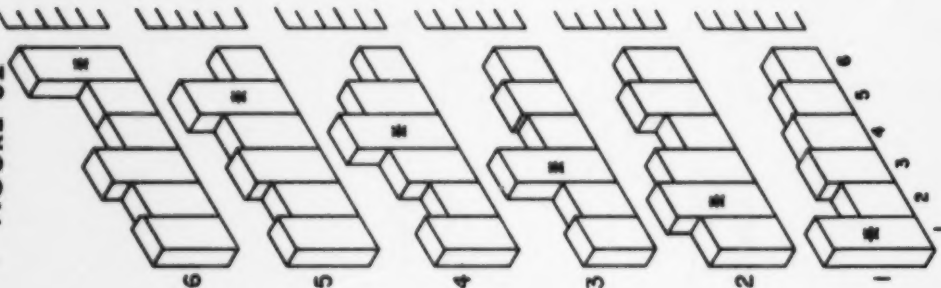
Table 53. - continued

H	I	J	K	Number of Isolates	Percent of total (1698)	Percent of 14 media on which appeared
4.5	3.0	2.6	3.3	269	15.8	100.0
2.1	12.2	2.1	1.7	288	17.0	92.9
3.6	5.9	2.7	6.8	221	13.0	92.9
6.8	13.0	1.9	6.2	161	9.5	92.9
3.4	6.8	3.4	1.7	117	6.9	92.9
8.9		3.0	7.9	101	5.9	85.7
10.0	3.0	1.0	3.0	100	5.9	85.7
5.4	13.5	2.7	2.7	37	2.2	71.4
5.9	5.9	2.9	5.9	36	2.1	85.7
3.1	9.4	6.3	3.1	32	1.9	85.7
3.3	3.3	6.7		30	1.8	71.4
5.3	5.3			19	1.1	71.4
8.3	4.2			24	1.4	64.3
3.7	14.8	7.4		27	1.6	57.1
6.9	3.4			29	1.7	50.0
7.7	23.1			13	0.8	50.0
8.3				12	0.7	50.0
14.3				14	0.8	42.9
		9.1		11	0.6	35.7
				11	0.6	21.4
				8	0.5	21.4
14.3	14.3			7	0.4	35.7
				5	0.3	28.6
				4	0.2	14.3
		50.0		2	0.1	14.3
				2	0.1	14.3
		2.0		34	2.0	35.7
7.7				13	0.8	21.4
9.1		27.3		11	0.6	42.9
11.1		44.4		9	0.5	35.7
	11.1	11.1	11.1	9	0.5	42.9
				8	0.5	14.3
				8	0.5	21.4
				3	0.2	14.3
				3	0.2	21.4
				2	0.1	14.3
				1	0.06	7.1
				1	0.06	7.1
				2	0.1	14.3
				2	0.1	14.3
				1	0.06	7.1
				1	0.06	7.1
				1	0.06	7.1
				1	0.06	7.1
33.3				3	0.2	21.4
	50.0			2	0.1	14.3
				1	0.06	7.1
				1	0.06	7.1
				1	0.06	7.1

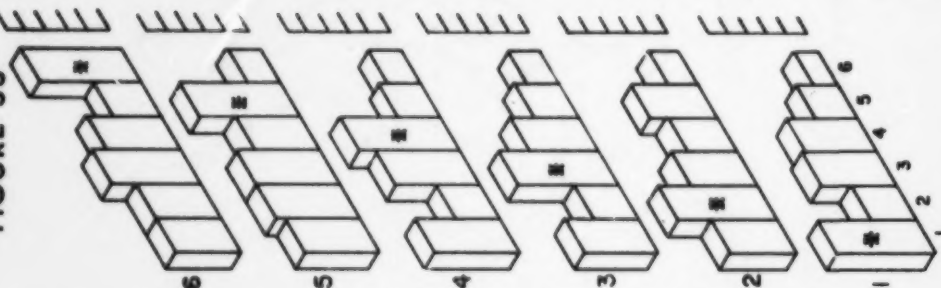
POND 1
FIGURE 31



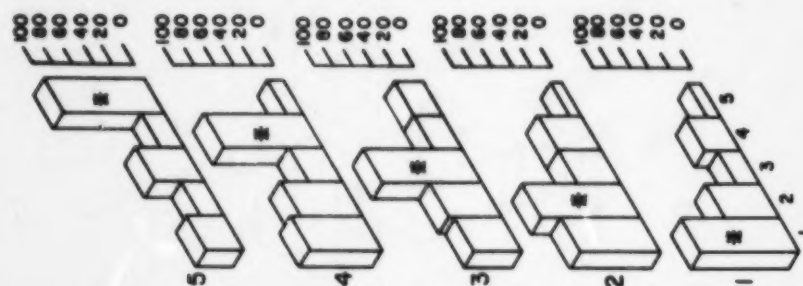
POND 2
FIGURE 32



POND 3
FIGURE 33



POND 4
FIGURE 34



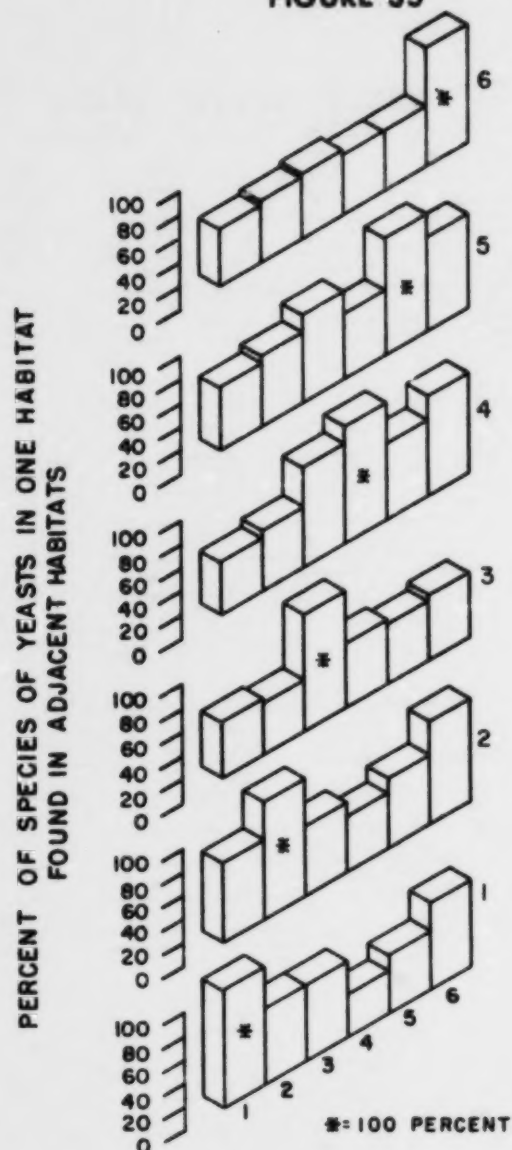
PERCENT OF SPECIES OF YISTS IN ONE HABITAT
FOUND IN ADJACENT HABITATS

100 PERCENT

LEGEND:

- 1 - RAW SEWAGE
- 2 - SLUDGE NEAR INFLUENT PIPE
- 3 - MID-POND WATER
- 4 - SURFACE SCUM
- 5 - SLUDGE NEAR EFFLUENT PIPE
- 6 - EFFLUENT

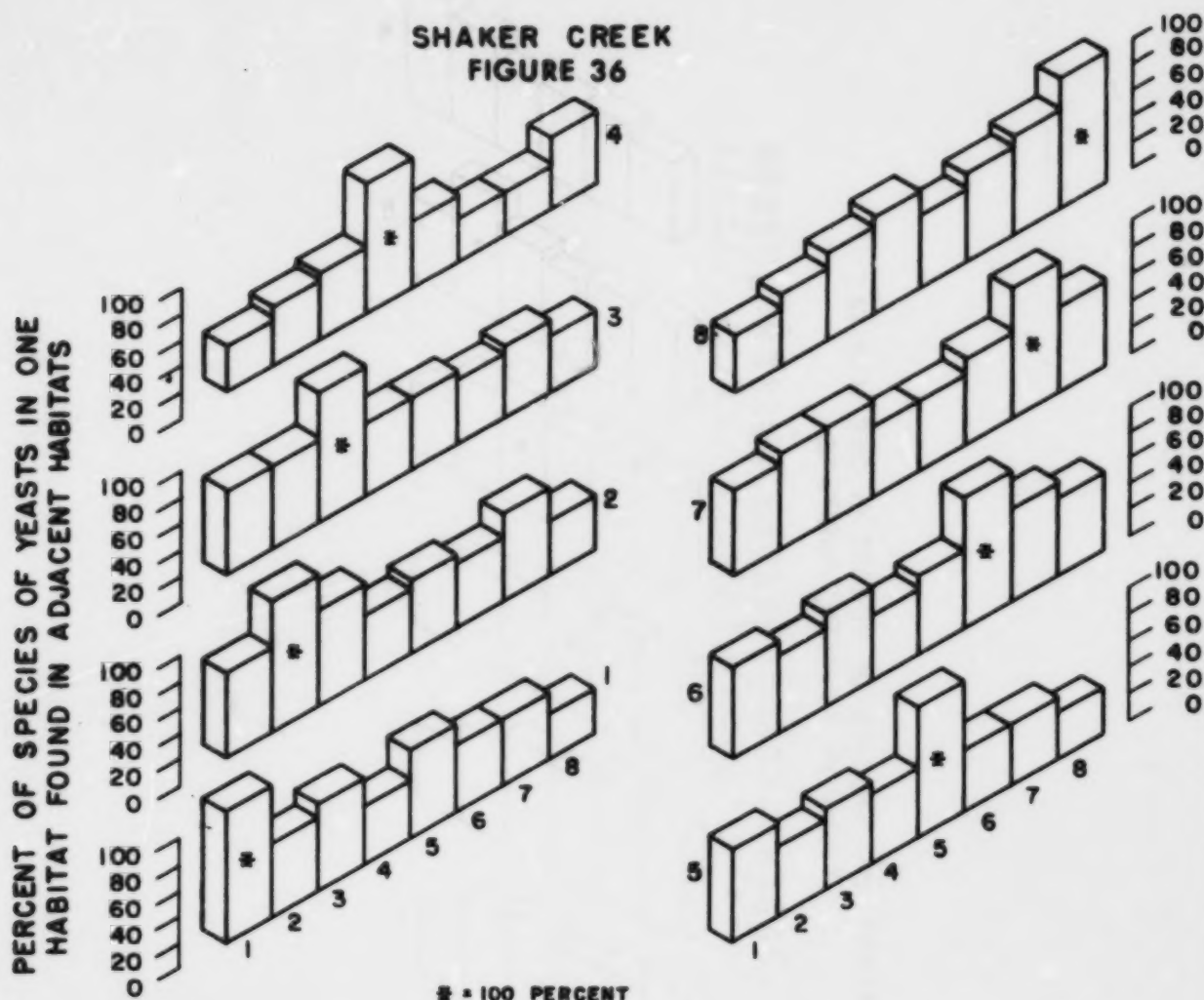
SOILS
FIGURE 35



LEGEND:

- 1.-UNDISTURBED SOIL
- 2-SOIL ON DIKE OF POND 1
- 3-POND 5: SOIL FROM CHANNEL CARRYING EFFLUENT FROM POND 1
- 4-POND 5: SOIL FROM CHANNEL CARRYING EFFLUENT FROM POND 2
- 5-POND 5: SOIL FROM CHANNEL CARRYING EFFLUENT FROM POND 3
- 6- POND 5: SOIL FROM MARSHY AREAS

SHAKER CREEK
FIGURE 36

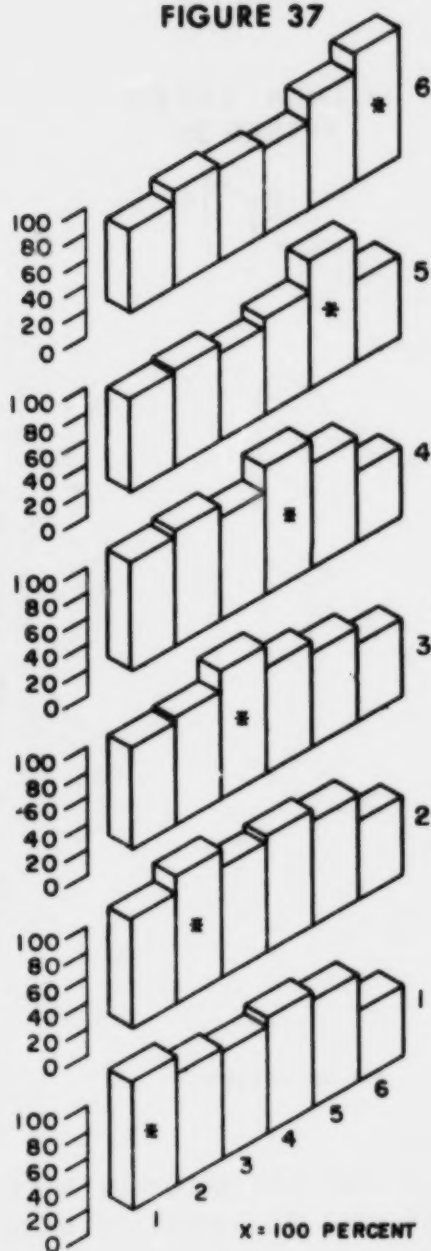


LEGEND:

- 1-UPSTREAM, WATER
- 2-UPSTREAM, WATER AND SEDIMENT IN RIFFLE
- 3-UPSTREAM, WATER AND SEDIMENT IN POOL
- 4-UPSTREAM, BANK SOIL
- 5-DOWNSTREAM, WATER
- 6-DOWNSTREAM, WATER AND SEDIMENT IN RIFFLE
- 7-DOWNSTREAM, WATER AND SEDIMENT IN POOL
- 8-DOWNSTREAM, BANK SOIL

SUMMARY
FIGURE 37

PERCENT OF SPECIES OF YEASTS IN ONE HABITAT
FOUND IN ADJACENT HABITATS



LEGEND:

- 1- POND 1
- 2- POND 2
- 3- POND 3
- 4- POND 4
- 5- SOILS
- 6- SHAKER CREEK

specialized segment of the total population, no analysis can be made of the dominance, relative abundance, or other ecological factors of this segment of the population.

Nutritional Considerations

With natural populations of higher plants, bryophytes, and even macrofungi and planktonic or benthic algae, the dominant members of the population can be related, directly or indirectly, to the nature of the parent material of the substratum and the climate of the region. One can expect to find a beech forest, a hemlock woods, or a prairie in a relatively predictable set of environmental conditions. In waters of streams whose environment has been altered by the addition of organic materials of one sort or another, certain changes take place, changes that are indicated by a change in the natural population. In a set of ponds that have been set-up artificially and whose populations are a direct response to the new artificial environment, whether the basic organisms are derived from the soil from which the ponds were made, from the living fraction of the organic matter, or from the air spora continually falling on the land and water, each developing situation must be described separately. Within the environments of sewage treatment processes, whether of the natural type in the reaches of a polluted stream or of the engineered type in the primary settling basin - preaerated or not, in the trickling filter or activated sludge tank, in the digesting or drying sludges, or in waste stabilization ponds, a special type of population has been selected. This selection may have started when the first human waste was added in small or even medium or large amounts to a relatively quiet stream,

and it may have continued to develop as the means of disposing of this waste became more and more complex, or it may have been able to develop momentarily in a place with the proper habitat factors. In any event, similar populations develop, with only the provision of an adequate habitat and food supply wherever human and organic industrial wastes are degraded in special treatment systems.

The 14 different types of isolation media and systems used in this survey each contributed some information to the total knowledge obtained to date on the yeast populations of these waste stabilization ponds. The fact that no two media, even when they were as nearly alike as YCB + $(\text{NH}_4)_2\text{SO}_4$ and YNB + glucose, or YNB + YCB, yielded the same segment of the population in the same amount indicates either that each ml of inoculum used contained a different assortment of cells or that these cells have different responses to conditions of the nutrient solution or to the protooperators or competitors associated with them in the inoculum.

Table 64 lists the various media, the number of species and colonies isolated thereon, and the percent of the total number of species and colonies these represent. Table 64 lists the number of isolates of each species, the percentage of the total number of isolates of each species obtained on each type of medium, and the percentage of the 14 media on which each species appeared.

A number of peculiar developments appear in this type of analysis. Among the routine tests of yeasts during identification procedures is a test for sugar utilization. A number of species that appeared on medium E, in YNB-lactose broth, are not able to utilize lactose as a sole carbon source in such

Table 64. Yeast colonies isolated from each medium.

Code Medium	Number of Species Isolated	Percent of Total Species	Number of Colonies Observed	Percent of Total
R Neopeptone-Dextrose-				
Rose Bengal-Aureomycin				
1 YNB + 1% glucose	22	40.7	173	10.2
20 YNB + 20% glucose	37	68.5	411	24.2
A YCB + (NH ₄) ₂ SO ₄	42	77.8	380	22.4
B YCB + NaNO ₂	23	42.6	80	4.7
C YCB + KNO ₃	2	3.7	8	0.5
D YNB + glucose	15	27.8	45	2.7
E YNB + lactose	17	31.5	67	3.9
F YNB + galactose	21	38.9	87	5.1
G YNB + maltose	17	31.5	62	3.7
H YNB + sucrose	21	38.9	86	5.1
I YNB + ethanol	23	42.6	79	4.7
J VYB	20	37.0	119	7.0
K YNB + YCB	17	31.5	42	2.5
	15	27.8	59	3.5
Total species isolated	54			
Total colonies reported			1698	

tests. A fairly large number of isolates were able to grow well in the presence of ethanol as the sole carbon source. Wherever more than one isolate of a species was obtained, it was isolated from more than one type of medium. No species appeared only on the agar dilution plates, and two species appeared only in the shaken flasks. On the other hand, five species were isolated from only one, two, or three of the more specialized enrichment media in still culture. Aside from undetermined species of white yeasts, only one species appeared on all 14 media used. This was Candida krusei, the most abundant species isolated in the survey. Oddly enough, in the description of Candida krusei presented by Lodder and Kreger-van Rij (1953), the species is reported to assimilate only glucose of the five test sugars and it does not grow on a medium containing nitrate as the sole source of nitrogen.

The facts that C. krusei and several as yet unidentified cultures of yeasts were the only strains obtained from the isolation medium containing only nitrite as a nitrogen source and that C. krusei, among other species, was isolated from other primary isolation media containing carbon sources not used by the organism in pure culture in laboratory experiments leading to the identification of these yeasts, would seem to indicate that other organisms were present that could alter the nutrients in these primary isolation media to the extent necessary to permit the growth of C. krusei. This is a type of synergism and may be referred to as protocoooperation (Alexander 1961). Such protocoooperative organisms must include bacteria and molds since some of the latter do utilize substrates and mold growth was present in the cultures, as will be indicated elsewhere.

It is interesting to note that only five species of yeasts were not obtained in the dilution plate and shake culture techniques and that of the

eight species not obtained in shaker culture none were very common species, one having been found in only three enrichment media on one occasion, four in dilution plate or enrichment media on one occasion each, and three in enrichment culture on only one occasion each. Although it is of interest that such species of yeasts are present in the habitat, and even though an occasional morphological or physiological novelty may be picked up in this way, it may be assumed by persons responsible for such surveys that species of such rare occurrence are not necessarily of importance in the systems under study.

Discussion

The yeast populations isolated from the waste stabilization pond system include species of sporogenous and asporogenous yeasts, some of which are pigmented, some of which reproduce only budding, others by budding and the production of pseudomycelium, and still others by the production, in addition, of true mycelium. In pure culture in the laboratory, various degrees of nutritional fastidiousness are observed and are used as taxonomic criteria in the separation of species within genera. Such nutritional restriction is not usually noted in primary isolations when the media include ammonia ions and glucose as the nitrogen and carbon sources, respectively, since N and C are available to all yeasts in these forms. When the primary isolation medium is prepared to exclude one or the other of these sources of nitrogen or carbon, however, certain species usually considered more fastidious appear to be able to develop in liquid still culture to such an extent that on the secondary agar isolation plates they appear in considerable number. Of course, it is possible that on Diamalt agar cells may

develop quickly to produce good growth after having remained dormant in the liquid culture for as long as 7 days. At least in the case of Candida krusei, however, and probably in the case of other species, activity on the part of those bacteria not affected by aureomycin and on the part of those filamentous fungi that developed was sufficient to release to the developing yeast population enough carbon or nitrogen sources in forms available to these organisms to permit relatively luxuriant growth.

In the populations of yeasts developed on a total of 14 media, 54 categories of yeasts were recovered from pairs of samples from 40 habitats in the four seasons of the year. Of these, 16 were found in undisturbed soils and 18 were found in the waters of Shaker Creek. No yeasts were recovered on neopeptone-dextrose-rose bengal-aureomycin agar from samples obtained from soils in 1957 and 1958 before sewage was applied to the bare ponds after the top 45-60 cm of soil had been removed for dike construction. This does not mean, however, that yeasts were not present since it has been shown that on this sampling medium throughout the eight successive series of samples only 20 species of yeasts were obtained. On the basis of other types of sampling, however, it seems unlikely that many colonies of yeasts were present in the subsoils on which the ponds were built. Those yeasts found in undisturbed soils may have formed an inoculum for the early populations of the ponds, especially if the pond waters reached the layers of the dikes in which top soils and accompanying yeast occurred. Because of the small quantity of sewage sampled at only eight times during the 2 years or more during which sewage was applied to the ponds, it is interesting that as many as 22 species were recovered. Aside from air-borne inoculum, the raw sewage formed the only

means of entry to the ponds for those species isolated from sewage, sludges, pond waters, scums, and effluents.

On the other hand, in Shaker Creek, continual flow from upstream could have brought to the sampling points inoculum from soils, barnyard wastes, and human and kitchen wastes. In the lower sampling area, additional fresh wastes from kitchen and barn could have added to those species already in the waters. Such inoculum under usual conditions of stream activity, could have settled along the creek in pools, riffles, and bank soil, and should sufficient organic matter have been present, developed into an active population rather than resting cells. Thus, the 29 species found in the creek were brought to the creek and influenced separately from those species found in the waste stabilization ponds, although most species recovered were common to both types of habitat. Because of seepage from the ponds, there was no direct overflow from the ponds to the creek, and because of the type of construction, there was no direct intake from the creek to the ponds, which at all times lay above the level of the creek bed.

It should be noted that in studies of the permeability of soil used in the sealing of the dikes forming Pond 2 creek water was used to test the ability of the pond to hold water. After it was found that the pond would hold water only to the 2-foot level, it was drained for further study. From the composition of the yeast populations that developed subsequent to sealing the walls with bentonite clay, it is apparent that this addition of creek water to the pond had no appreciable effect on populations observed during the sampling periods.

The influence of the flow system within the ponds on the yeast populations is best indicated by the figures demonstrating fidelity. Since these

figures are based on total populations without regard to number of samples removed or season of sampling, they aid in pointing out areas where fewer samples could be obtained, especially in connection with pond effluents. Figs. 31 through 34 show that within the ponds the sludges contained fewer yeast species than the liquid and surface habitats sampled. Since the ponds, e.g., Pond 4 (Fig. 34), lost more and more of their water through seepage or evaporation or both, rather than through normal overflow, the yeast populations declined in the effluent, which in some cases could not be sampled. Fig. 35 shows that as less and less effluent reached the channels in the bed of Pond 5 from Ponds 2 and 3 the habitat was affected by their effluent less and less.

Population analyses presented in Figs. 24 through 30 show that within the pond habitats yeast populations developed best in such liquid phases as mid-pond waters. Within soil types, yeast populations developed better in areas where greater organic enrichments of soils occurred. Both types of analysis (Figs. 29 and 36) show that more yeasts occurred in riffle sediments, but fewer species occurred at the lower sampling station than at the upper. In neither sampling area was bank soil very rich in organic matter. This soil was usually composed of tan clayey muds developed on top of the blue shales characteristic of the Ordovician limestones of the area. Such muds were washed away during and after each high water resulting from heavy precipitation and therefore formed a poor habitat for the development of a good population of many types of microorganisms.

On the basis of the wide variety of media on which colonies developed and the number of organically enriched habitats from which these species

were isolated, it may be said that within this system of waste stabilization ponds, i. e., in the soils influenced by correlated organic enrichment and in the adjacent stream influenced by similar materials, the following species were able to develop, indicating the influence of such enrichment: Candida krusei, C. tropicalis, C. parapsilosis, Rhodotorula glutinis, R. mucilaginosa, and Saccharomyces cerevisiae.

So far we have found no clinical record of disease in those who contribute to the organic enrichment of these habitats that would indicate a relationship between the inoculum producing the populations of the first three species and conditions of intestinal disorder. In current texts on medical mycology these species are not considered to be related to such disorders in the temperate regions of the world, but in tropical countries certain disorders have been attributed to the action or the existence of these yeasts in the human body. It should not be surprising to find large populations of red yeasts in any natural areas, and to find that such populations have been influenced by enrichment of waters. The cultures of Saccharomyces cerevisiae developed most abundantly in November samples, although the organism was found as early as October and remained in continuing lesser amounts in February and March. This development may have been related to the use of baker's yeast in the kitchen that supplied the mess hall with "home-baked" bread. The Saccharomyces carlsbergensis cultures picked up on three occasions may have come from the same source.

In addition to the six species mentioned above, the presence of certain other species may be considered to demonstrate the presence of excessive amounts of organic enrichment of the habitat under special, as yet incompletely

defined, conditions. These species appeared with sufficient frequency to represent 1.5-2.2% of the total number of isolates recovered from samples tested. They include: Candida guilliermondii, C. curvata, Torulopsis famata, T. glabrata, T. inconspicua, Trichosporon cutaneum, Cryptococcus albidus and C. laurentii. It is of interest that strains of all these fungi have been isolated from human materials, but that none is associated with truly diseased conditions of man.

It is noted that under the conditions of this survey no strains of the two commonest yeasts known to produce disease of man in this region were isolated: Candida albicans and Cryptococcus neoformans. It is thought that these species could have grown in some of the media used.

The occurrence of a wide variety of species of yeasts from a number of genera, in habitats such as these, indicates that yeasts are ubiquitous organisms with rather general nutritional requirements that can be satisfied throughout the year in a number of habitats, especially where organic enrichments may be present. That previously they have been isolated largely from clinical materials, animal tissues, substrata related to the food and beverage production industries, and the bodies and habitats of such organisms as shrimp and fruit-flies, only indicates areas of interest in relation to special study projects. As identification procedures become better known, the search of additional habitats for these organisms may increase our knowledge of their distribution and importance in nature.

Summary

From 40 habitats within a waste stabilization pond system for the treatment of sewage from a small institutional dormitory and dining hall,

samples of sewage, sludges, pond waters and effluents, and undisturbed and disturbed soils were collected. From these samples and from samples of materials from an adjacent stream, 54 categories of yeasts were isolated in a year-round survey. Although all of the commoner species have been isolated from human clinical materials in past work, none of the species found has been associated with human disease conditions in temperate regions of the northern hemisphere.

All of the commoner and some of the less-common species were isolated from shaken cultures in YNB broth, with 1% and 20% glucose used as paired media. Other species, as well as all the commoner species, were obtained on broths made of YCB and YNB media to which were added different nitrogen or carbon sources. For routine surveys of populations the shaken YNB broth cultures seem adequate, but quantitative techniques must be developed for more critical delimitation of populations.

In general, within the sets of samples tested, Candida krusei, C. tropicalis, C. parapsilosis, Rhodotorula glutinis, R. mucilaginosa, and Saccharomyces cerevisiae appear indicative of organic enrichment of waters and soils.

VIII. Fungi in Sludge Digesters

In the above described work with materials from sewage, sewage-polluted water and sewage treatment systems, it has been shown that fairly large numbers of filamentous fungi and yeasts can be recovered regularly. Depending on the media used, and possibly on the antibiotics or growth rate retarders, the number of fungal cells in digester sludge was lower than that in sludge from an Imhoff tank or a primary settling basin, but this number increased rapidly as the sludges dried out on open-air drying beds. Retention time was usually about 40 days, and numbers of colonies recovered from sludges in the bottoms of the digesters reached the hundreds of thousands per milliliter. It was pointed out that a broad spectrum of fungal species occurs in digesting sludges, and that as some species declined in number, others increased during the digestion process.

An experiment by others in the Basic and Applied Sciences Branch, Division of Water Supply and Pollution Control, Robert A. Taft Sanitary Engineering Center, Cincinnati, made available interesting samples of digesting sludges for examination for fungus cells or spores.

Procedures

Two digesters, similar to those used by McDermott et al (1963) were operated during the first experiment. Digested sludges resulted from the following operations: A slurry of fish meal was prepared and mixed with raw sewage. This was settled in a flow-through tank, and the resulting primary settled sludge was added daily to one digester as daily samples

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were removed for determination of quality and activity. A second portion of the settled mixture of raw sewage sludge and fish meal was fed to an activated-sludge system, from which excess secondary sludge was removed. The excess activated sludge was mixed with the previously described primary sludge, and this mixture was fed to a second sludge digester. Both were operated as continuous digesters, with a detention time of 16 days. The digesters produced an average of approximately 700 milliliters of gas per gram of volatile solids fed. Reduction of volatile solids was satisfactory.

A sample of each of the primary settled sludge and the mixed sludge, and samples from each of the two digesters were examined as described in detail above. Six isolation methods were used: (1) direct pour plating for colony counts on neopeptone-dextrose-rose bengal-Aureomycin agar (NDRBA) at 1:1000 dilution, (2) Sabouraud broth on a shaker using 1% glucose at a 1:10 dilution, (3, 4, 5) yeast-nitrogen base (YNB-Difco) broth on a shaker using 1% and 20% glucose in separate media and dilutions approaching extinction of 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000; and (6) processing the samples, in the case of the last 8 sets, at the five mentioned dilutions using membrane filters incubated on YNB-glucose and NDRBA agars. In addition to enumerating colonies and cells, species of all types of fungi were picked for identification from the first four sets of NDRBA plates, from all shaken flasks, and from membrane filters.

Growth of fungi as demonstrated by their recovery on poured agar plates and in shaken flasks was sufficient to warrant further study designed to determine whether the fungi studied were survivors, or a

growing population in the substratum in which they were found. After the digested sludges had come to equilibrium as demonstrated by the production of gases, and the condition of the sludge after one complete turnover, or 16 days time, one digester was fed with autoclave-sterilized fish meal slurry. The second digester was fed with a nonsterilized fish meal slurry. If fungi were present as resting cells only, that is, if fungi were doing nothing in the digestion process, then progressively fewer species and colonies would be recovered from the sludge.

The samples obtained from the second experiment differed from those used in the first experiment to the extent of the differing feeds from the time the use of sewage feed was discontinued and the digesters were fed only with sterilized and unsterilized fish meal. Since sampling was started when fish meal slurries were first added to digesters in which sewage sludges had been brought to equilibrium with the digestion process, the four samples contained residual sewage sludges. As the experiment proceeded through more than seven complete turnovers with sustained digestion, the total initial population was reduced by a factor of about 3,000. Techniques of study of these samples remained the same except that Sabouraud broth was not used, and the membrane filter technique was used throughout the series of 14 sets of samples observed. In all cases these techniques were strictly aerobic. A list of fungi recovered in both experiments is given in Table 65.

Results

Preliminary evaluation of fungal populations. Between June 10 and July 26, 1962, 31 sets of samples of primary settled and digested sludges

Table 65. Species of fungi observed in a study of sludge digestion.
Experiments I and II combined

Species	Observation Number	Sample dates of 44	Sample Areas of 8
<u>Mucor hiemalis</u>	635	41	129
<u>Geotrichum candidum</u>	606	44	142
<u>Candida krusei</u>	237	23	43
<u>Moniliaceae</u>	177	23	54
<u>Penicillium</u> spp.	160	36	82
<u>Trichosporon cutaneum</u>	121	30	60
<u>Torulopsis candida</u>	73	20	38
<u>Candida tropicalis</u>	64	18	40
<u>Torulopsis farnata</u>	63	20	31
<u>Phialophora jeanselmei</u>	62	18	51
<u>Dewatiaceae</u>	54	13	26
White Yeasts	51	22	32
<u>Trichoderma viride</u>	51	25	41
<u>Cladosporium cladosporioides</u>	47	16	28
<u>Aureobasidium pullulans</u>	47	19	33
<u>Aspergillus niger</u>	45	12	18
<u>Torulopsis glabrata</u>	42	17	28
<u>Rhodotorula mucilaginosa</u>	41	18	30
<u>Rhodotorula texensis</u>	40	14	22
<u>Candida parapsilosis</u>	29	16	21
<u>Fusarium</u> spp.	29	9	13
<u>Candida catenulata</u>	25	16	19
<u>Cephalosporium</u> spp.	20	12	18
<u>Candida intermedia</u>	18	11	12
<u>Rhodotorula pilimanae</u>	18	9	14
<u>Penicillium lilacinum</u>	18	14	17
<u>Torulopsis</u> sp.	17	7	12
<u>Alternaria tenuis</u>	17	9	13
<u>Candida humicola</u>	16	7	12
<u>Trichosporon capitatum</u>	14	10	12
<u>Candida curvata</u>	12	8	11
<u>Phoma</u> spp.	12	5	9
<u>Kloeckera apiculata</u>	11	8	10
<u>Candida mycoderma</u>	10	6	8
<u>Mucor fragilis</u>	10	5	8
<u>Candida guilliermondii</u>	9	4	4
<u>Cladosporium herbarum</u>	9	4	5
<u>Torulopsis inconspicua</u>	7	4	6
<u>Epicoccum purpurascens</u>	7	6	6
<u>Penicillium chrysogenum</u>	7	4	6
Smut-like	6	4	4
<u>Aspergillus flavus</u>	6	4	5
<u>Fusarium oxysporum</u>	6	4	4
<u>Rhizopus</u> spp.	6	6	6
<u>Candida rugosa</u>	4	4	4
<u>Aspergillus fumigatus</u>	4	2	4
<u>Monosporium apiospermum</u>	4	4	4
<u>Mucor racemosus</u>	4	3	4
<u>Candida albicans</u>	3	2	3
<u>Candida isolani</u>	3	2	2
<u>Torulopsis</u> sp.	3	2	2
<u>Trichosporon pullulans</u>	3	2	2
<u>Prototheca</u> sp.	3	1	1
<u>Aspergillus sydowii</u>	3	3	3
<u>Aspergillus tamaris</u>	3	2	2

Table 65. p. 2

<u>Aspergillus</u> spp.	3	2	3
<u>Candida japonica</u>	2	2	2
<u>Cryptococcus albidus</u>	2	1	1
<u>Rhodotorula glutinis</u>	2	2	2
<u>Saccharomyces cerevisiae</u>	2	1	2
<u>Torulopsis seria</u>	2	2	2
<u>Torulopsis dattila</u>	2	2	2
<u>Torulopsis holmii</u>	2	2	2
<u>Penicillium ochrocloron</u>	2	2	2
<u>Periconia</u> sp.	2	2	2
<u>Peyronellea</u> sp.	2	2	2
<u>Phoma herbarum</u>	2	2	2
<u>Aspergillus amstelodami</u>	2	2	2
<u>Paecilomyces varioti</u>	2	2	2

The following species were observed only once in this series of samples:

Candida mesenterica, C. utilis, Saccharomyces fructuum, Torulopsis carpophila,
Aspergillus terreus, A. versicolor, Botryotrichum piluliferum, Fusarium aqueductum,
Paecilomyces sp., Penicillium expansum, P. martensii, P. tardum, P. variable,
P. waksmanii, Phialophora sp. Scopulariopsis brevicaulis, Stachybotrys atra,
Subbaromyces splendens, Verticillium sp., V. lateritium.

were processed. These were collected at daily intervals, Monday through Friday, for the seven weeks of the sludge digestion experiment, except on July 4. Illness of a staff member prevented processing of samples on three days. Since theoretical detention time was 16 days, the test period represents three complete turnovers in each of the two sludge digesters.

The total number of species or species groups isolated from the samples surveyed was 66. Figure 38 shows the percent of these species obtained on each sampling date. There is a gradual decline in number of species throughout the duration of the experiment. At the beginning of the experiment a wider variety of species is represented; but of the 66 species, 15 were found fairly constantly throughout the series, and this group of species included many of those found more frequently toward the conclusion of the experiment. Thus a population adapted to survival or growth in the peculiar environmental conditions of the sludge digester was in the process of being selected. Its member species were being replaced less frequently by invader species in the later periods than in the formative stages in the earlier part of the experiment.

The average numbers of colonies of fungi recovered on NDRBA plates during each of the 7 weeks are plotted in Figure 39. Samples #1 and #2 show a larger number of fungus colonies going into the system than were being recovered from the digested sludges. When samples #1 and #3, and #2 and #4, are paired, except for numbers of colonies, there is a close correlation between populations of settled and digested sludges of the two types. even though the sludge represents a 16-day accumulation.

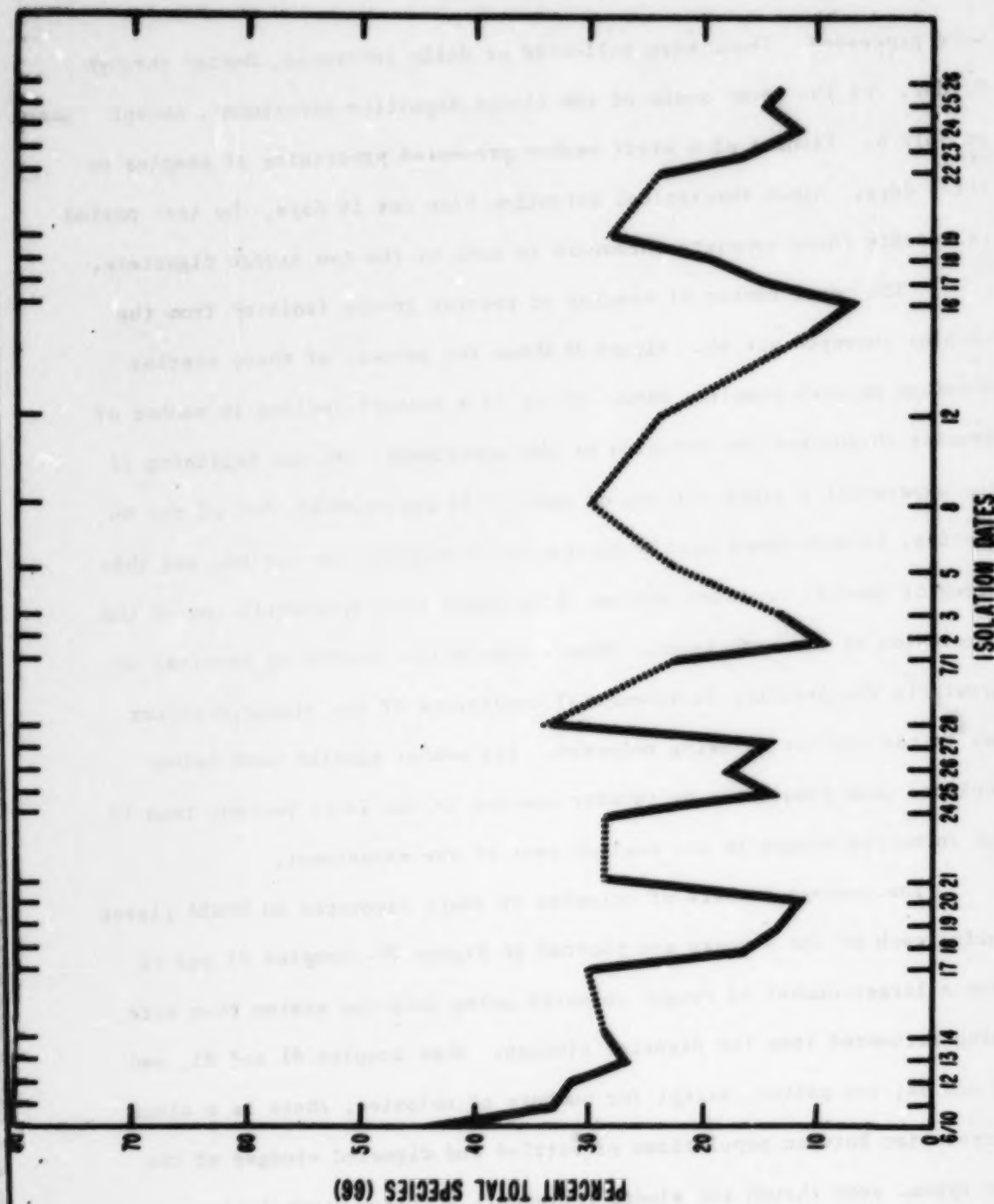


FIGURE 38. PERCENT OF TOTAL (66) SPECIES OF FUNGI ISOLATED ON EACH SAMPLING DATE, EXPERIMENT I.

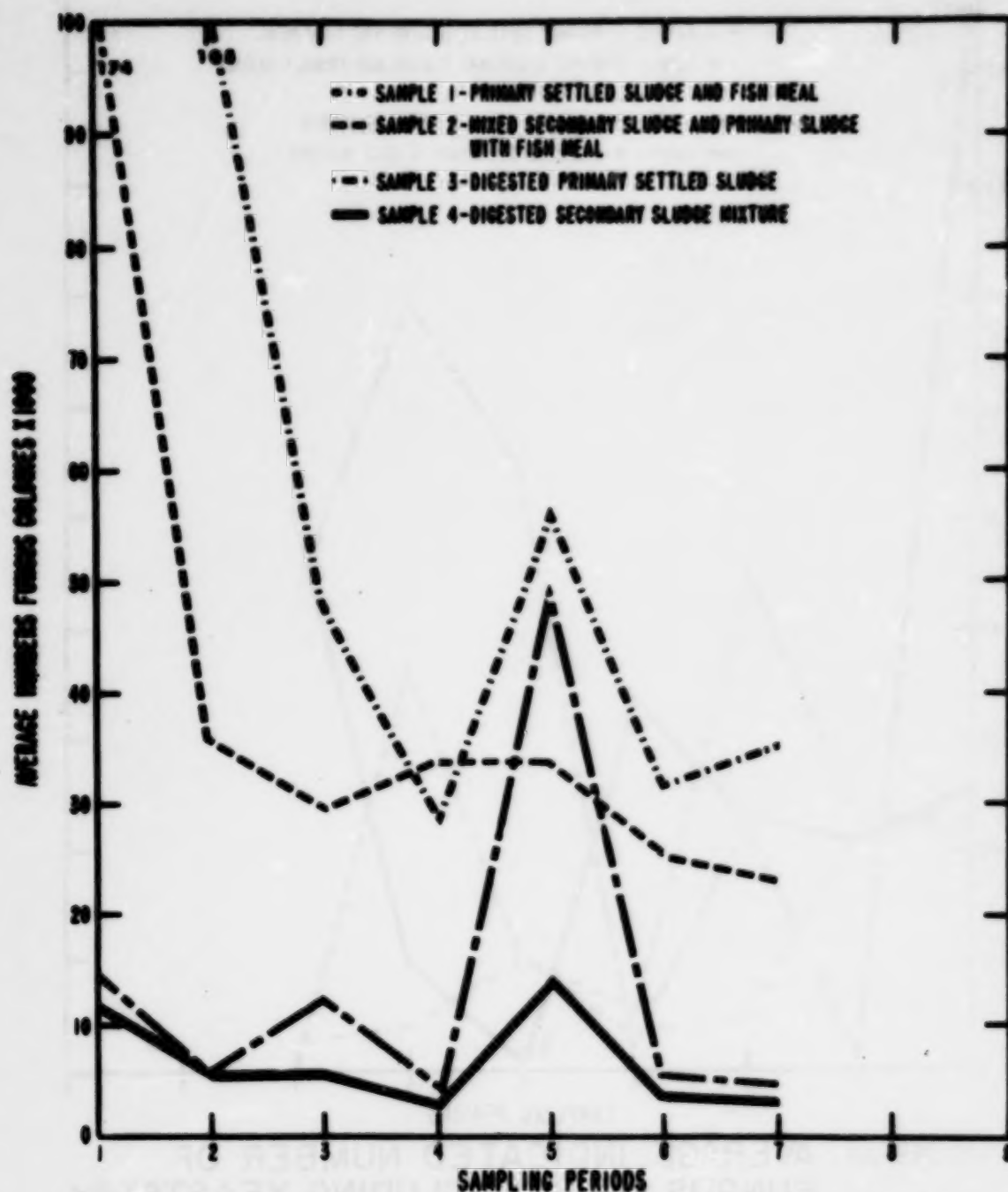


FIGURE 39. AVERAGE NUMBERS OF COLONIES OF FUNGI RECOVERED PER ml SAMPLE ON AGAR PLATES X 1000. EXPERIMENT I.

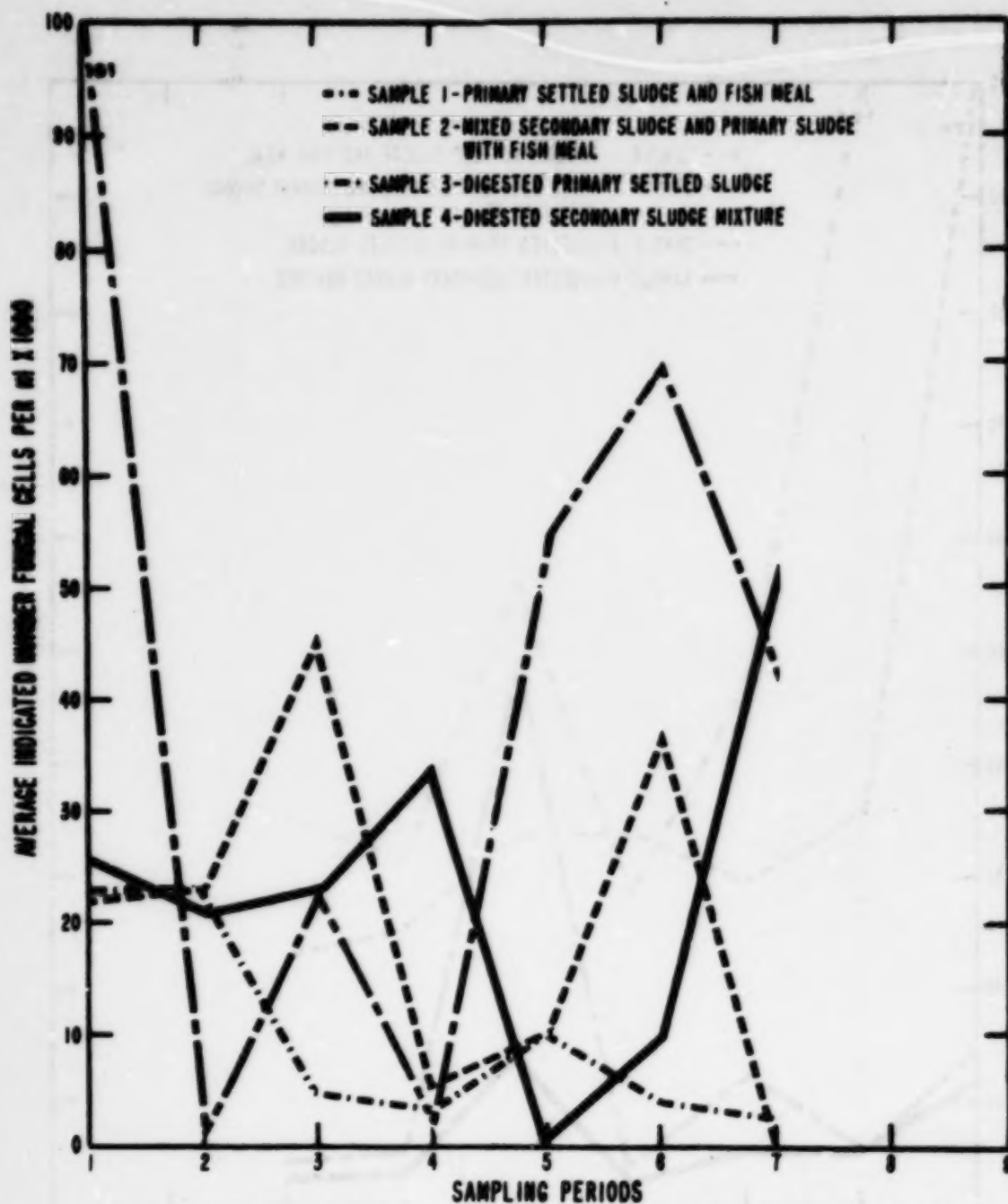


FIGURE 40. AVERAGE INDICATED NUMBER OF FUNGUS CELLS (INCLUDING YEASTS) BY INSPECTION FOR EACH PERIOD PER ml SAMPLE X 1000. EXPERIMENT I.

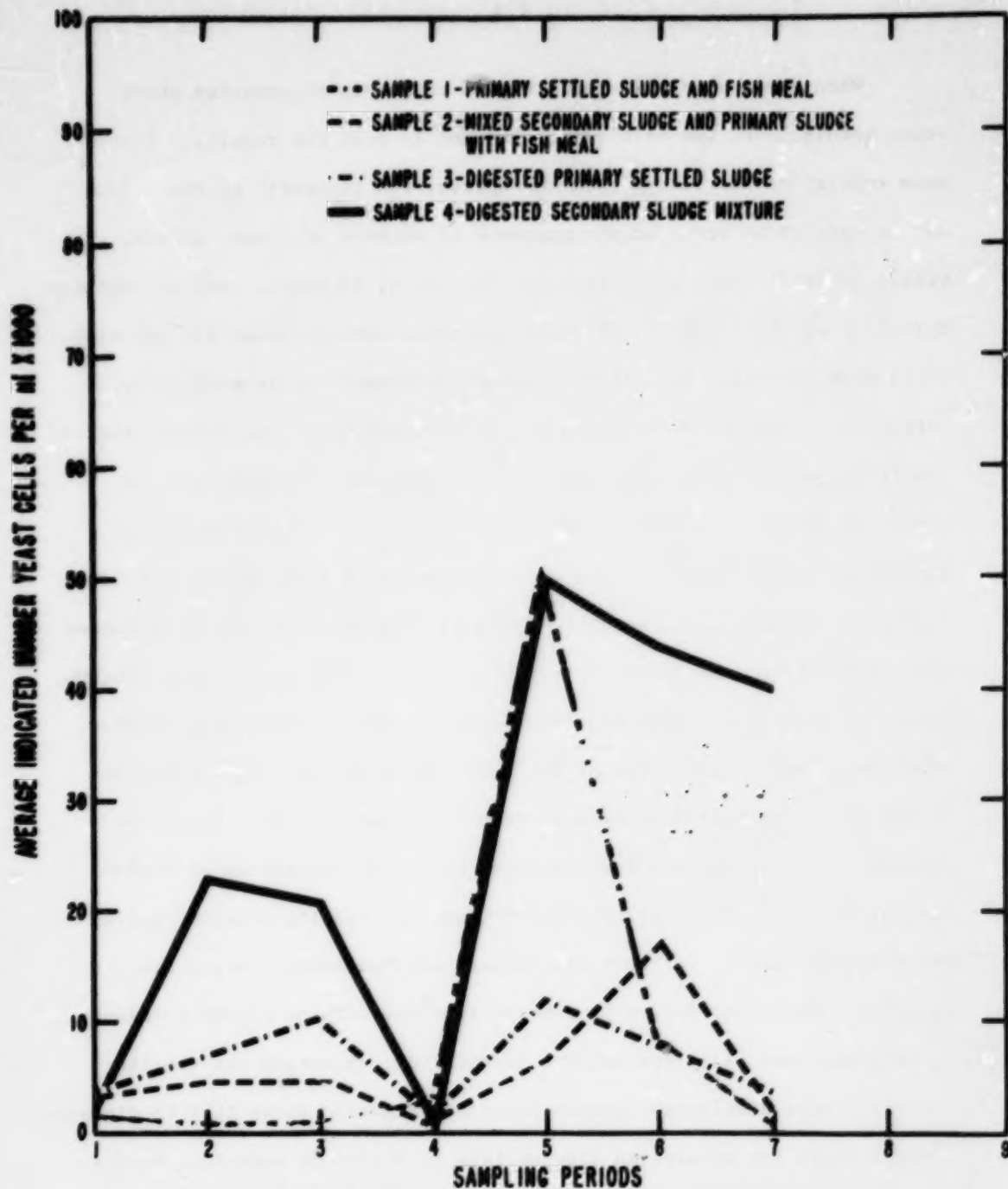


FIGURE 41. AVERAGE INDICATED NUMBER OF YEAST CELLS BY SPECIES FOR EACH PERIOD PER ml SAMPLE X 1000. EXPERIMENT I.

When enrichment cultures are used to obtain information about yeast populations, two methods may be used to read the results. First, upon removal of the flasks from the shaker, the turbidity of the medium may be used as an index of the presence or absence of fungus growth. If viable cells of fungi are present in the one ml of sample used as inoculum, turbidity in the 1:100,000 dilution indicates that at least 100,000 viable cells were present. This is the "indicated number" as determined by inspection. Second, such growth may be streaked onto agar plates, the resulting growth sorted and purified into component species, and the number of species recovered from each dilution may be translated into numbers of cells per milliliter of inoculum (see Figures 40 and 41). If 6 species are recovered from the 1:100,000 dilution, the indicated number per milliliter of sample based on recoverable species is at least 600,000. Values on which the graphs are based were obtained by averaging results from the 1:1000 dilution flasks for each weekly period. Again, samples #1 and #3 may be paired as well as samples #2 and #4. When indicated numbers of cells (Figure 40) are studied merely by inspection of shaken flasks, there is little correlation between the results obtained during the 7 weeks. Curves for both the primary and secondary sludges are erratic. When yeast growth is removed from the complex growth obtained in this way, and after species are identified, replotting the results reveals a more distinctive growth pattern. Figure 41 shows that in digested sludges there are population fluctuations in which, on occasion, yeasts are numerous in sludge digestion, and also, more numerous in the digested

sludges than in the raw sludges. In the development of the data for Figure 41, the filamentous fungus, Geotrichum candidum, was screened out. The erratic curves in Figure 40 are largely attributable to the growth of this fungus.

Fungal populations of non-sewage sludges. Samples from the second experiment, in which sterilized and non-sterilized fish meal were added routinely to balanced digesters, were brought to the laboratory once a week between October 4, 1962, and January 4, 1963. The seventh week was omitted in November, 1962, and a final check was run on February 20, 1963, before the completion of the experiment. Seven theoretical detention periods were covered by this series of samples. At first, fish meal slurries were autoclaved for one-half hour at 20 lb pressure. Sterilization was not found to be complete until the slurries were left in the autoclave for at least one hour.

In this series of samples, 41 species of fungi, including molds and yeasts, were found. The largest percentage of these was found on the 28th day, the smallest on the 42nd, as shown in Figure 42. By the 139th day, after the start of feeding the digesters with fish meal, with one digester fed sterilized fish meal, fungi were still present in both digesters. Also, in both digesters the species present were members of a population that apparently was rapidly becoming stabilized. Since the only sewage added to the systems was that used to initiate them, the only source of new members of the populations was the nonsterilized fish meal, with a population presumed to have been constant, added to the second

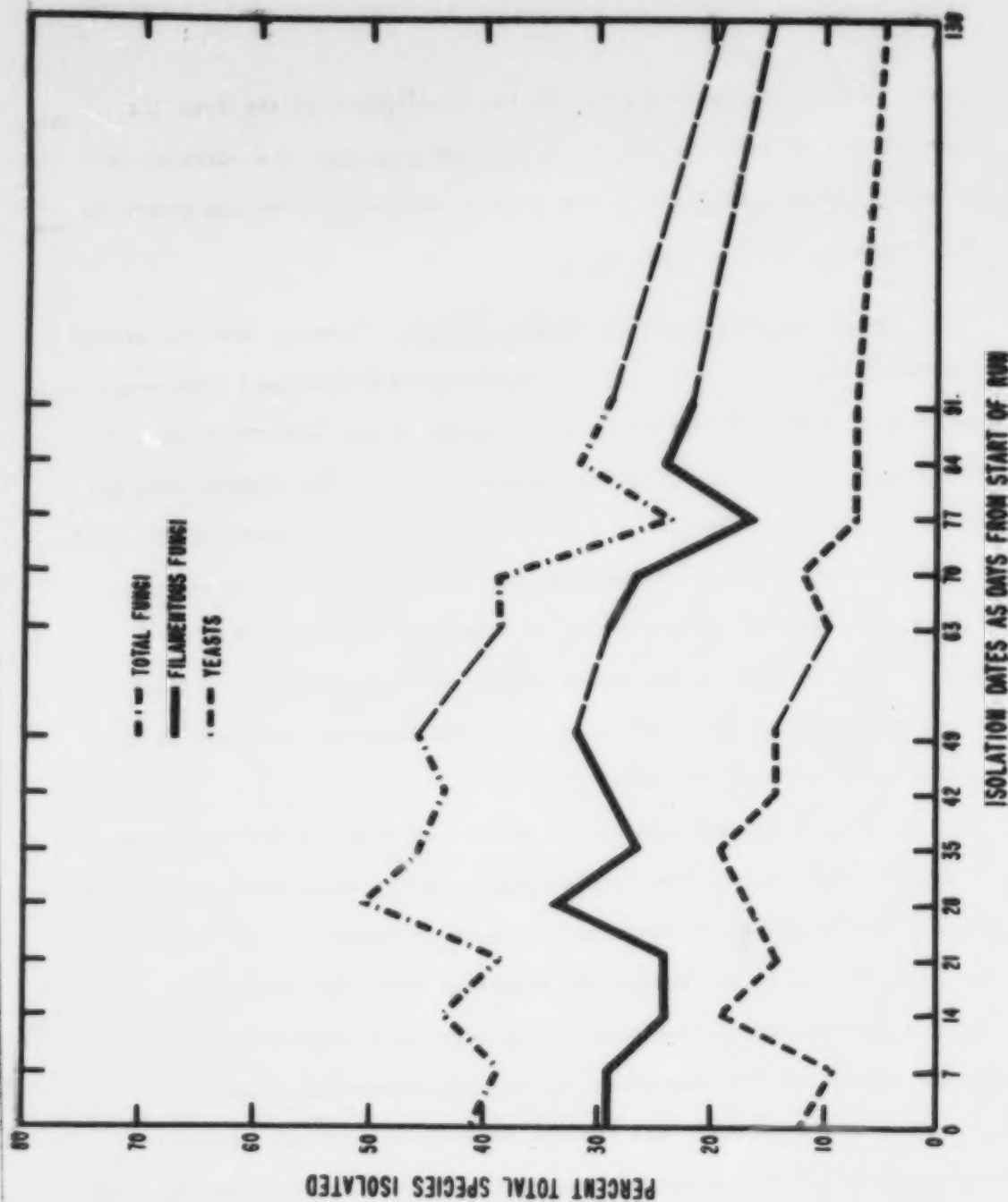


FIGURE 42 . PERCENT TOTAL (41) SPECIES ISOLATED FROM
EXPERIMENT 2 SAMPLES.

digester; the sterile fish meal slurries added to the first digester contributed no new members to the population.

Numbers of colonies of fungi recovered on NDRBA agar plates during the course of this experiment are shown in Figure 43. In this figure, samples #2 and #4 are paired, while sample #3 has no matching line, the sample of sterilized fish meal having no initial population. Data for sample #3 indicate the population carryover from the initial three weeks of operation, during which a sludge digestion population was built up in the process of bringing the digester to the equilibrium of efficient operation. Since the digesting fish meal sludge progressed satisfactorily, it must be presumed that the population of fungi found in sample #3 was carrying out metabolic processes in a sludge undergoing digestion. The same must be presumed for other members of a population characteristic of sludge digestion.

Two techniques are used to show minimal indicated numbers of cells of fungi in the settled and digested sludges used in the second experiment. In Figure 44 the average indicated number per milliliter of fungus cells of all types recovered from the four types of media used is shown. Here, samples #2 and #4 are roughly parallel although during the week in which the Thanksgiving holiday occurred there was a sharp decline in numbers of cells in the digested sludges, which started to recover during the following week. Sample #3, from the digesting fish meal slurry, likewise showed a slump at that time, but this was less drastic and recovery was better.

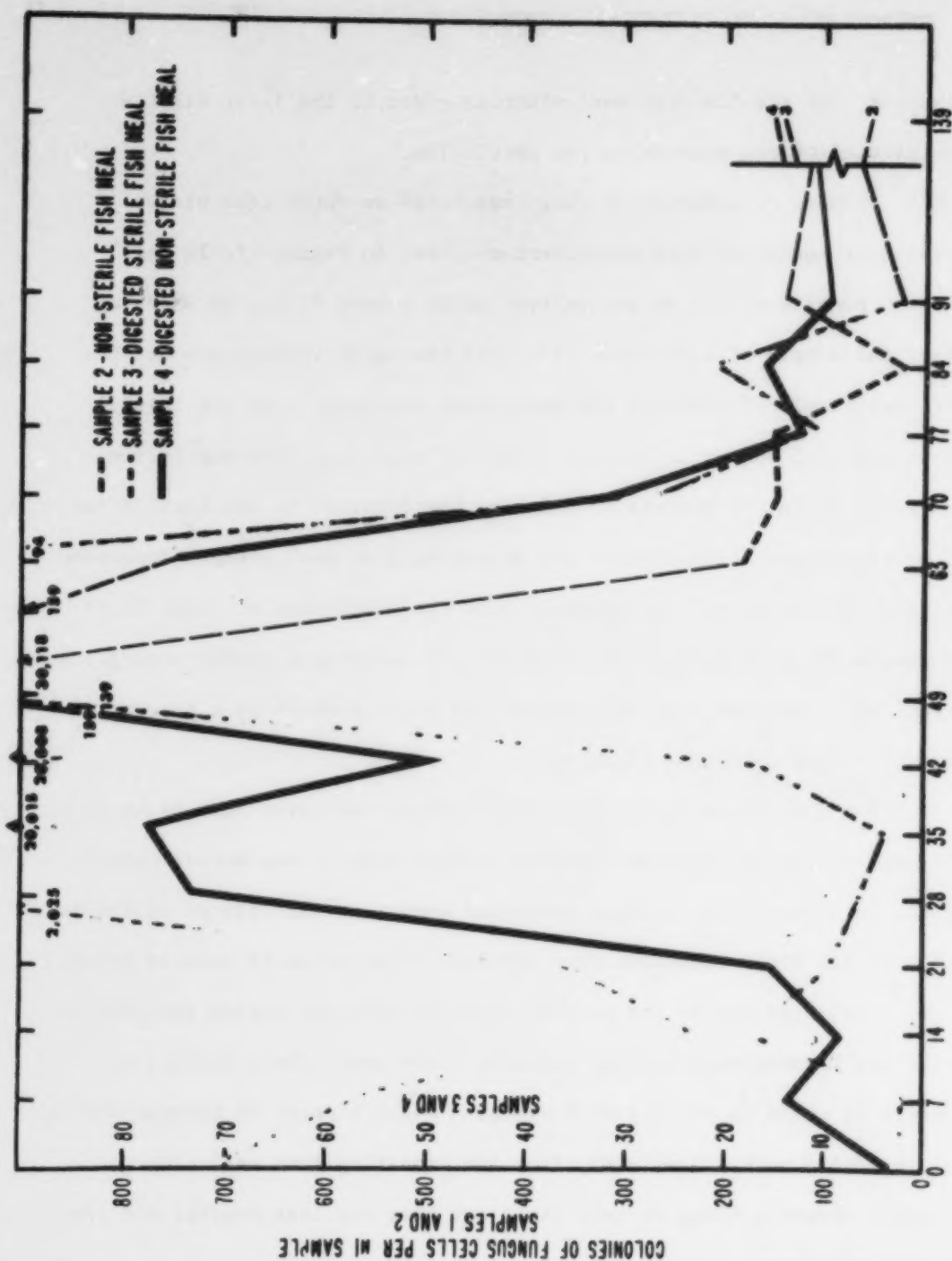


FIGURE 43. NUMBER OF FUNGUS CELLS PER ml OF SAMPLE
BASED ON COLONY COUNTS ON AGAR PLATES.
EXPERIMENT 2.

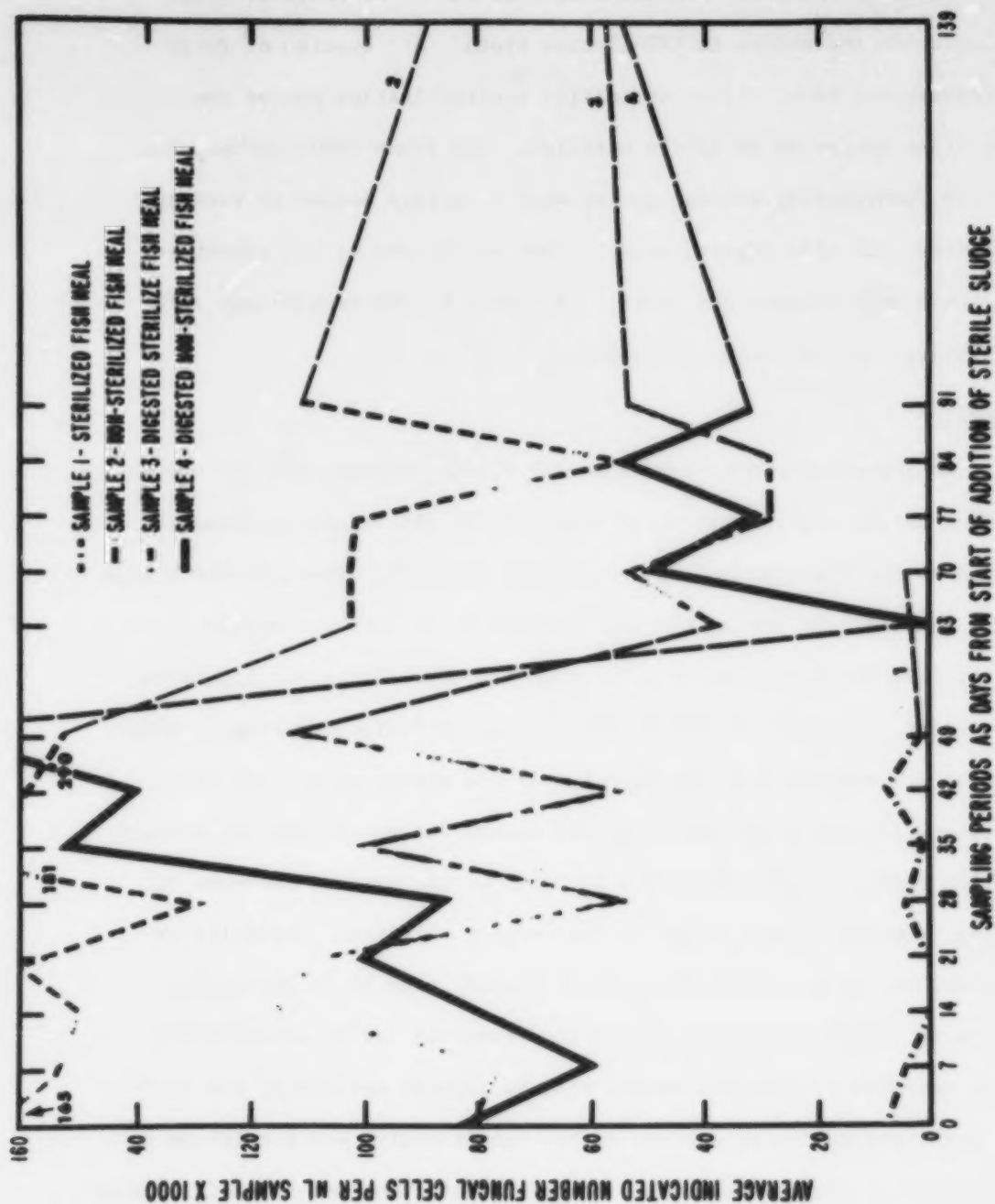


FIGURE 44. AVERAGE INDICATED NUMBER OF ALL FUNGUS CELLS PER ml SAMPLE FROM ALL MEDIA X 1000 EXPERIMENT 2.

Figure 45 shows the minimal indicated number of cells of fungi developing on the shaker in YNB-glucose broth. All species of fungi are represented here. After an initial acclimatization period the three lines appear to be fairly parallel. The slump shown corresponds with the Thanksgiving holiday period with a certain amount of recovery thereafter. In this figure, growth shown at the end of the experiment represents only filamentous fungi, the yeasts having dropped out after the 91st day of the second experiment.

Discussion

In the usual processing of sewage sludge, primary settled sludge is added to the digester at brief intervals or relatively continuously as long as the digester has space to accommodate it. The digested sludge may be removed from the bottom of the digester as the theoretical detention time is reached or as more space for more sludge for digestion is required. Sewages contain a wide variety of living organisms. Some of these are removed with the detritus as the sewage enters the plant; others are increased in numbers as the sewage passes through the primary aeration tanks, if such tanks are included in the system; and some are removed from the liquid sewage during primary settling. These latter organisms are added to the populations already present in the sludge digesters. Those that adapt to the conditions of low or unmeasurable oxygen supplies in a liquid medium rich in organic materials, and inorganic salts and solids of various sorts, remain active and contribute to the process of sludge digestion. In the process they add to their tissues and form cells that can reproduce the parent. Spore production, as it is

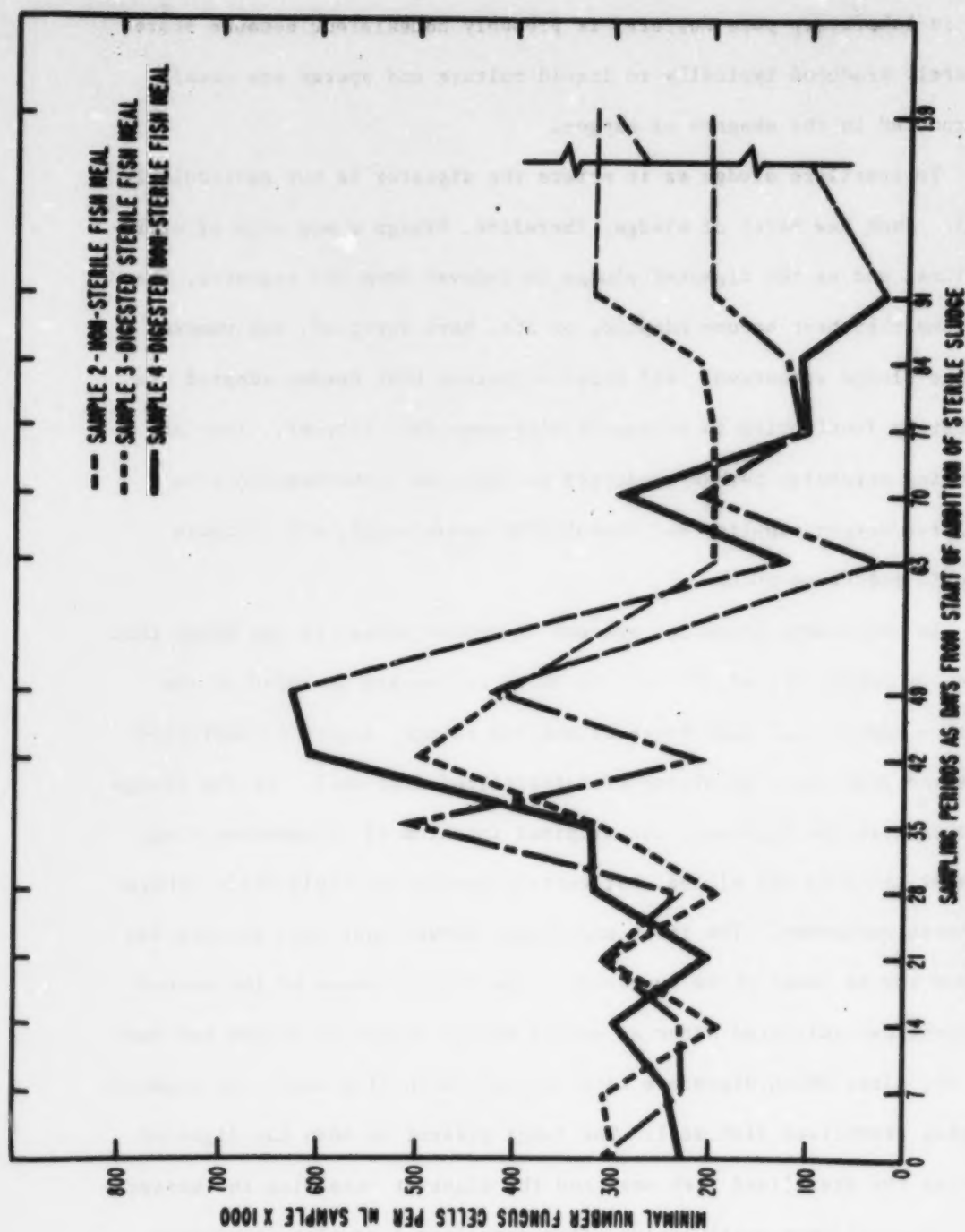


FIGURE 45. MINIMAL NUMBER OF CELLS OF ALL FUNGI PER ml SAMPLE BASED ON INDICATED NUMBER IN SHAKEN FLASK CULTURE YNB-GLUCOSE BROTH X 1000 EXPERIMENT 2.

known in laboratory pure culture, is probably nonexistent because spores are rarely produced typically in liquid culture and spores are usually not produced in the absence of oxygen.

To sterilize sludge as it enters the digester is not particularly useful. Each new batch of sludge, therefore, brings a new crop of microorganisms; and as the digested sludge is removed from the digester, the organisms that have become adapted, or that have survived, are removed with the sludge withdrawn. All those organisms that become adapted are necessarily functioning in an essentially anaerobic habitat. They are thus using metabolic pathways adapted to chemical transformations in which free oxygen supplies are essentially nonexistent, and obligate anaerobic processes occur.

In the sludge digestion systems described above, it was shown that a sizeable population of filamentous fungi and yeasts is added to the digestion system with each batch of settled sewage, slurry of activated sludge and fish meal, or slurry of unsterilized fish meal. As the sludge passes through the digester, the original inoculum of filamentous fungi and yeast cells is not killed, but certain species multiply while others may remain quiescent. The first experiment showed that this process can continue for at least three turnovers. The initial phase of the second experiment was initiated after an active sludge digestion system had been built up, after which digesters were fed only with fish meal, one digester receiving sterilized fish meal. The fungi present in both the digester receiving the sterilized fish meal and the digester receiving the unsterilized fish meal were still active after more than 7 complete turnovers

(see Figures 42 through 45). If these fungi had been inhibited by the changed conditions in the digesters, their numbers would have decreased as the digestion periods increased. However, by the end of more than 7 complete turnovers, when only fish meal was added as a substitute for sewage sludge, or even for sewage sludge fortified with fish meal, certain fungi no longer survived, although others took their place. The order in which the yeasts appeared to drop out indicated that the species capable of fermentative reactions, producing alcohol and carbon dioxide, were among the first to go, while the filamentous species, incapable of fermenting sugars, persisted. These fungi included species not previously known to be able to survive and multiply under conditions of almost complete anaerobiosis, as measured by the amount of dissolved oxygen in the habitat. It is of interest to note that fish meal is proteinaceous in nature, and the final populations of filamentous fungi found in these digestion experiments include no species known to be actively cellulolytic. Normally developed sewage sludges include high percentages of cellulosic materials.

The deep depressions in the curves for both sets of experiments coincide with holiday periods. Sampling period 4 in Experiment 1 includes July 4, while the 46th day in Experiment 2 includes Thanksgiving Day. Both periods resulted in brief interruptions to the draw-and-feed cycle, which could easily have interrupted the rhythmic development of the populations under consideration. Sudden interruptions in the receipt of fresh food supplies could easily disrupt metabolic patterns and reduce the numbers of cells producing colonies or growth in recovery media.

Conclusions

Fungi, including yeasts and molds, are present in the process of the digestion of sludges in sewage treatment systems. These organisms can be demonstrated by their culture from samples of sludges in a variety of media. The approximate minimal number of cells present in digesting sludges can be determined. After three complete turnovers of digesting sludges with a retention time of 16 days, the yeasts and molds still present in considerable numbers in daily draw-out samples, indicate that they may be taking part in the digestion process to the extent of obtaining nutrients for growth, rather than being merely present in the quiescent state.

Following the development of a stable sludge-digestion system, many yeasts and molds are present. The use of sterile and nonsterile fish meal as a substitute for sewage solids furnished an adequate substrate for the growth of filamentous fungi and for maintenance of a sludge, which was still adequately digesting after more than seven complete turnovers.

Fungi, including yeasts and molds, are members of a population of miscellaneous microorganisms occurring in sludge digesters. They have been adequately demonstrated in laboratory scale digesters as well as in operational sludge digesters.

The presence of large numbers of viable cells at the end of more than seven complete turnovers, even though the total initial population was reduced by a factor of about 3,000, indicate that filamentous fungi can grow and multiply in an anaerobic digester. Whether they are an important factor in digestion has not been determined.

IX Fungi in Relation to Lignin and Paper Mill Wastes *

A. Natural and Induced Fungal Degradation of Lignin

Lignin in fossil plant materials. In the natural disposal of the remains of plant and animal materials, two types of activity occur: degradation and preservation. If plant materials, in which we are primarily interested, are produced near an inland shallow body of fresh water, die, and fall onto the wet land or into the water, they may decompose partially through aerobic processes and partially through anaerobic processes. If they become submerged meanwhile, aerobic decomposition will cease and anaerobic degradation may continue. As more and more such materials accumulate, anaerobic disintegration may discontinue in the deeper parts of the bog, leaving a partially decomposed mass of trunks, roots, stems, branches, twigs, and leaves subject to lignification. This mass of material includes lignin, cellulose, and waxy and resinous materials of many types from many sources. Through the ages this peat may become brown coal, lignite, soft coal, anthracite, and even possibly graphite, through the several processes of coalification outlined by Schopf (1948).

It appears that in the Paleozoic Period, 250 to 300 million years ago, during which the carboniferous strata were laid down, considerable anaerobic decomposition of plant materials took place in the formation of peat. But as time went on these anaerobic processes were at least partially replaced by aerobic processes as indicated by the type of degradation found in coals developed during the Cretaceous (125,000,000 years ago) and Tertiary (60,000,000 years ago) periods. In addition to types of decay,

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the finding of fungal remains in the latter but not in the earlier coals (Schopf, 1952), lends further credence to the idea that aerobic processes replaced anaerobic processes in the decomposition of materials comprising the peat. This may be correlated with the fact that later bogs were more estuarine than inland in nature.

In dryer areas that may be found near inland lakes and coastal estuaries, the type of preservation was entirely different and is known as petrification. Here a tree or a forest may have become covered with a layer of sand or volcanic ash with resulting infiltration of cells of wood and bark with sand particles. The conditions of burial here, and in shallow ponds where layer upon layer of silt or ash covered increments of leaf and fruit deposit, prevented any decomposition, and the infiltration or impression left a detailed record of the regional vegetation. Following infiltration, various types of material were left such as petrified or agatized wood. Where such materials are formed, disturbance to their tissues is evident only as burned tissue, heart rot, and surface damage by fungi and insects, such as may have occurred prior to complete embedding in the preserving stratum.

The fact that cellulose is removed in part but not completely from woods of as late an age as the Tertiary leads to the supposition that anaerobic decomposition as well as aerobic decomposition occurred, the former by anaerobic cellulose-decomposing bacteria and the latter by cellulolytic fungi. The habitat in which submerged materials accumulate in peat bogs would preclude the action of fungi which are known to be, at least today, aerobic in habitat requirements. Both aerobic and

anaerobic organisms may be active in decomposing cellulose not completely submerged, and both fungi and bacteria would be active.

On the basis of the low percentage of the recognizable cellulose present, lignin may be presumed to be the primary constituent of coal (Barghoorn, 1952a, b). From our present scanty knowledge of the organisms associated with lignin and cellulose degradation in nature, we may assume that anaerobic and aerobic bacteria and fungi have had the capacity for cellulose degradation for almost as long as cellulose has been a part of the numerous products of plant metabolism. Because of a lack of oxygen in the habitat, it may be further assumed that under the conditions of the peat-bog-type of habitat, lignin-degrading organisms are not active. Certainly other plants existed when the coal measures were being built up, and grew in areas near but not influenced by conditions responsible for petrified forests. Since there is no trace of these plants, cellulose and lignin-degrading organisms were probably present in those areas when the materials were available for degradation.

Lignin in living plant materials. Apparently lignin is not associated with plant material at evolutionary levels of the Thallophyta and the Bryophyta. However, with the development of vascular tissues, lignin, which appears to aid in mechanical stiffening of plant tissues, is found. It occurs in all parts of the plant from roots through stems or trunks to leaves. In the rye plant, temperature of germination and growth appears to control the amount of lignin formed (Ghumann, 1950). Depending on the plant part or type of cell, the lignin content varies. The middle lamella

in wood cells appears to contain large percentages, while in the remainder of the cell wall lignin or lignin precursors are mixed with cellulose to a greater or less degree (Sharp, 1943). Lignin is rare or absent in non-vascular plants, but it or similar material is reported in the fruit bodies of certain perennial wood decay fungi as well as in other fungi (Foster, 1949). It appears in conifers (except Ginkgo) (Johansen, 1940) in a different form than in hardwoods, according to stain reactions, color reactions in the presence of certain indicator chemicals, and differences in industrial by-products containing lignin.

From such work as that of Roadhouse and MacDougall (1956), it appears that the formation of lignin deposits in plants takes place slowly and that only in mature tissues do lignin or lignin compounds occur.

Chemical degradation of lignin. Brauns, 1952, has reviewed the chemistry of lignin, its isolation, chemical identification, its degradation, some chemical reactions and by-products, how it can be removed from forest products, and how it can be used to the advantage of industry.

Biological degradation of lignin. In recent years a number of types of studies have been carried on in an effort to find organisms that will degrade lignins. Usually such studies have noted the growth of organisms such as fungi in lignin-containing liquids, have investigated whether certain fungi or bacteria will utilize lignin as their sole carbon source, or have studied the resulting byproducts with a view to determining the degree and type of degradation that has occurred. Studies

have been concerned with litter and humus (Broadfoot and Pierre, 1939), soils (Norman, 1936), decaying litter, decaying wood in the forest, sawdust compost, spent sulphite liquor, and other substrata in which microorganisms live and appear to utilize lignin compounds. The organisms concerned are usually fungi, but bacteria have also been considered. Work with agricultural soils and early analyses for lignin have not been included here, since techniques have been generalized or analyses have not been as critical as are presently required in the study of lignin and compounds resulting from its degradation.

Much of this type of work has been summarized in a literature survey prepared (Lawson & Still, 1956) for the use of the employees of the West Virginia Pulp and Paper Company. It is inevitable that a certain amount of duplication would occur between their summary and mine, and in studying their summary, certain references which had been overlooked were brought to my attention. Biological degradation of lignin takes place by the activities, in general, of two types of organisms. Bacterial degradation need be mentioned only briefly here, since it is generally highly controversial, and little work has been done in this direction. Certain soil, rumen, and termite-associated bacteria are involved, but the extent of such involvement is still incompletely understood. Activities of fungi in several ways will be considered in more detail.

Mycological degradation of lignans. In his book on humus, Waksman (1938) described the lignin problem concisely and listed only certain wood-decomposing fungi, certain species of Coprinus, and the common mushroom, Agaricus campestris, as capable of decomposing lignins in plant materials.

Throughout the book, various problems of lignin decomposition or accumulation are discussed or briefly mentioned. Waksman and his students (1936) have shown that as lignin content of plants increases, the ability of microorganisms in the soil to decompose them decreases.

Recently, specific work has been carried on in which the relation between fungi and the lignin components of litter and decaying wood has been considered. In the United States this has been limited to gross studies of loss of weight as a result of degradation of all parts of the litter by whatever microorganisms are present in an incubated sample on a long-term basis (Broadfoot and Pierre, 1939). Others have carried on similar research, but where the fungi active on lignin components were not identified the work will not be cited here. In the following paragraphs, fungi associated with composts, litter, wood and lignosulphonates will be treated separately. The uses of fungi to isolate lignins for chemical analyses will be indicated.

Composts. It has long been known that the common mushroom (*Agaricus campestris*), as well as other fungi which have been found on manure and decaying wood, can utilize lignin as a source of carbon. Composted manure is used in the mushroom industry as the principal food source for the common mushroom. Waksman and Nissen (1931) showed that this fungus used lignins and complex nitrogenous compounds preferentially in its growth on composted manure. Gottlieb and Geller (1949) were able at least partially to purify and study an enzyme from mushroom spawn which, apparently unlike other phenol oxidases, catalyzed a lignin oxidation reaction.

Humfeld (1948) and Humfeld and Sugihara (1949, 1952) found that mushroom mycelium could be produced in good quantity in submerged shaker culture. The nutrients for optimal development were determined as well as those for optimum development of a mycelium which had an odor and flavor very near those of the mushroom grown on compost beds. Certain wild mushroom species (Sugihara and Humfeld, 1954) were also grown in this way with little modification of the basal medium. While no attempt was made by these workers to develop lignin utilization by such cultures, Szuets (1953) reported that he could produce edible mushroom mycelium using spent sulphite liquor in unused penicillin vats. It was suggested that this type of use could tide a penicillin producer over a period of slump in the antibiotics market.

In Japan, Higuchi (1953) found an enzyme in mushroom spawn which oxidized the low molecular portion of Brauns' native lignin.

Sawdust composts, treated with anhydrous ammonia and phosphoric acid, were used in seed beds and pots containing seedlings of conifers and deciduous trees and of medium red clover. Davey (1953, 1955) incubated the fungus Coprinus ephemerus in composts developed from jack pine, sugar maple and red oak sawdusts. It was found that after 8 or 16 weeks the lignin content of jack pine sawdust had decreased, while that of the other two was increased, although the reverse of this was true of the lignin methoxyl groups.

Litter. Beech leaves and pine needles were used by Falck (1930) in a study of the activity of Agaricus (Clitocybe) nebularis, a litter dweller, and Coniophora cerebella, a dry rot fungus. It was found that

these fungi represent two basic groups: those that split off lignin leaving acid humus, represented by the Coniophora, and those that break the lignin and consume the humus, represented by the Clitocybe.

In Sweden, Lindeberg has been concerned with the decomposition of lignin and cellulose in litter. Soil-inhabiting, litter-decomposing, and parasitic species of Hymenomycetes have been the principal organisms whose activities were considered. Twenty-six species of litter- and wood-decomposing fungi (Lindeberg, 1946) were inoculated on undecomposed dead beech leaves, and of these 13 broke down more than 30% of the dry matter and 15 decomposed more than 50% of the lignin in the leaves. Seven species were inoculated on straw of the grass Glyceria maxima, and of these 5 broke down more than 30% of the dry matter and these same species decomposed more than 65% of the lignin. The cultures were maintained stationary at 25°C for 160 to 174 days.

Eighteen species of fungi known to produce, or suspected of producing, mycorrhizae with forest trees; 26 litter-decomposing species known to attack both cellulose and lignin; one species known to attack cellulose; and 17 untested litter-decomposing species; were tested on gallic and tannic acid media for their polyphenol oxidase content (Lindeberg, 1948). Four mycorrhizal species and many litter-decomposing species were found to possess o-diphenyl oxidase and one was found to secrete this enzyme into the medium. This was thought to indicate the ability of these fungi to use the lignin of the litter as a food source.

Lindeberg (1948) regarded lignin as a product resulting from condensation of building units closely related to the o-diphenols, and he

stated that units of the same type are found in tannins. On this basis, the fact that nutrient solutions in which certain litter-destroying Hymenomycetes have been grown contain o-diphenol oxidase or catecholase indicates that such fungi may be capable of breaking down lignin. Catecholase produced in culture media by four litter-decomposing fungi and two parasitic fungi was studied for effects of temperature. It was found that at 60°C the activity of the enzyme was inhibited only to a very limited extent, although temperatures as high as 69°C inhibited its activity. Potassium cyanide and potassium azide strongly inhibited the activity of the catecholase produced by Marasmius graminum. The optimum pH of activity of the catecholase from each of the fungi tested compared favorably with the pH of the natural substrata of the fungi.

Fourteen species of Marasmius were studied by Lindeberg (1944) for their ability to decompose litter, their natural habitat, and for their physiological activities. It was found that after 6 to 7 months all but one species had reduced the lignin content of pine needles by 48 to 59%, of beech leaves 67 to 72%, and of straw by 62%. Only those species naturally occurring on a given substrate were used in testing similar substrata.

In a summarizing review of work done with litter and wood-decay fungi, Lindeberg (1956) described the ability of litter-decomposing and mycorrhiza-building fungi to use lignin in comparison with cellulose, to produce a positive polyphenol oxidase reaction in culture media, and to produce laccase or tyrosinase in culture, in the fruit body, and in the mycelium.

Wood. It has been shown by extensive experimentation that many wood decay fungi require thiamine as a vitamin supplement in the media in which they are grown. Since cell wall material is a nitrogenous compound, and protoplasm contains numerous compounds of which nitrogen is an important part, supplemental sources of nitrogen are required by wood decay fungi. The enzyme systems of most, if not all, fungi contain both exo- and endoenzymes. Polyporus (Hirschioporus) abietinus has been shown (Garren, 1938), to produce at least 15 hydrolytic and four oxidizing enzymes, while Zeller (1916) has shown that Lenzites sepiaria produces at least 21 enzymes. The former species is a white-rot, the latter a brown-rot of conifers. Wolf and Wolf (1947) tabulate 24 enzymes and the known ability of 24 species of wood-decay fungi to produce them. Garren's work was confirmed by Lyr (1955). He studied 103 fungus species of which 43 excreted laccase, 13 peroxidase, and the remainder no oxidases. Peroxidase formers live in heartwood and prefer oak and fir. No heartwood destroyers restricted to cellulose formed oxidases. If not strict cellulose destroyers, sapwood-using species usually formed laccase.

As early as 1899, Hartig (1899) attributed the destruction of lignin in wood to the activity of Fomes (Porodaedalea, Trametes) pini.

Two distinct types of wood decay were reported by Falck and Haag (1927) as a result of the activity of Polyporus (Fomes, Fomitopsis) annosus, which partially destroyed the lignin in pine wood, producing a "corrosion" type of rot, and Merulius domesticus (Serpula lacrimans), which largely utilized the cellulose, leaving the lignin intact and producing a "destruction" type of rot. The lignin content decreased in the first type, and was "built up" in the second on a percentage basis.

Findlay (1940) found in comparing the rate of activity of brown-rot, Coniophora cerebella, and white-rot Polyporus (Coriolus) versicolor, fungi that the latter could completely destroy blocks of beechwood within two years in pure culture under controlled conditions. The former left as much as 70% of the wood substance in the same length of time under the same conditions. Polyporus versicolor apparently first removed the lignin and then utilized the hydrocarbons.

Heuser et al. (1949) found that Polyporus (Hirschioporus) pargamensis used the lignin of aspen wood. Blocks of the wood were attacked with varying intensity as indicated by color zones: of the tan, pink and white zones, the white zone was most heavily attacked. The presence of pentosans seemed to favor attack on the cellulose portion of the wood.

Twelve wood-rotting fungi, known to use lignin since they produced a white-rot type of decay, were tested for their ability to utilize various types of phenolic compounds (Fähraeus, 1949). Oxidation zones were not produced by five substances that inhibited growth and 16 that were not or only slightly inhibiting. White or yellow zones were produced by seven substances that more or less inhibited growth. Eight very toxic phenols gave dark brown to violet or nearly black zone lines; 13 less toxic phenols and 10 aromatic amines also gave dark zone lines. The enzyme laccase was suspected present although it was not isolated in the pure state. Some of these substances appear to be useful for isolating lignin-decomposing fungi from soil and wood, and may be useful in studying humus. Alpha-naphthol at concentrations of 10 to 100 ppm permits growth of lignin-decomposing fungi but not others, while at 70 ppm it inhibits some germination presumably because spores do not contain any phenoloxidase.

Using sawdust, ^OFähraeus, Nilsson and Nilsson (1949) investigated the ability of three wood-decay and litter-decomposing fungi to use cellulose and lignin. After 6 months a high percentage of original material was used. Polyporus abietinus and Marasmius scorodoni were found to prefer lignin while Stereum rugosum preferred cellulose but could use lignin. Pure cellulose could be removed from fresh sawdust only in part and was much more easily removed after decay by the lignin-using fungi.

Two white-rot fungi, Polyporus (Coriolus) zonatus and P. versicolor, were found to form a laccase-type of phenol oxidase extracellularly (Lindeberg and ^OFähraeus, 1952). This was intensified with increased pH or with the addition of potassium iodide to permit only a slow growth of mycelium.

^OFähraeus (1952) found that the production of laccase by Polyporus versicolor in artificial media varied with the type of medium. In the nonsynthetic medium, malt extract, the enzyme was formed and secreted into the medium early in the growth period, although it was rapidly inactivated in this medium. On a synthetic medium this production was delayed and a high production activity was not reached until autolysis of mycelium occurred. Several possible reasons for this are based on constituents of the medium and the liberation of various types of cell components in the autolysis.

In further work with P. zonatus and P. versicolor (^OFähraeus and Lindeberg, 1953), it was found that an increased production of laccase occurred in artificial media to which tyrosine and four other amino-acids

and aromatic compounds were added. The enzyme oxidized none of these compounds but eventually produced an inhibition of growth, possibly because of toxicity. Excess production of the enzyme might favor a reduced synthesis of other cell constituents.

Polyporus versicolor and Fomes pini were grown on spruce and birch sawdust for 6 months by Henderson (1955). Vanillic acid was released from both sawdusts by both fungi but syringic acid was released only from birch sawdust. This confirms chemical analyses that both hardwood and conifer lignin contain guaiacyl groups, but syringyl groups are found only in hardwood lignins.

In pure culture sterilized spruce and birch wood were attacked by six species of fungi in studies by Meier (1955) on cell-wall breakdown. After treatment of the degraded wood samples, microtome sections were studied under the electron microscope. Cellulose fibrils and arrangement of cellulose and lignin fractions could be studied with different degrees of effectiveness after action of the several fungi on the cell wall materials.

Savard and Andre (1956) studied the effect of Poria sp., a brown-rot, and of Lentinus nigripes, a white rot, on the wood of Triplochiton scleroxylon. The Poria was most destructive, since it removed cellulose and pentosans but apparently allowed lignin to accumulate, while Lentinus removed lignin, cellulose and pentosans at about the same rate.

Lignosulphonic acids. In 1942 research was started in Canada in an attempt to find fungi capable of using lignin and thus reducing the lignin wastes of the paper industry. Adams and Ledingham (1942a) found that the blue stain fungus, Endoconidiophora adiposa, could decompose nearly 10% of the lignin in spent sulphite liquor after 20 days in still culture,

although in nature stain fungi are not supposed to be able to degrade lignin (Gaeumann, 1950). Incubation temperatures to 25 to 30°C, different sources of carbonates as neutralizing agents, and different sources of nitrogen were used. Ammonia and organic nitrogen sources were found to be best, sodium carbonate was found to be a good neutralizer, and relatively high pH levels were required. Consistent results were readily obtained. Lignin utilization ended shortly after most of the carbohydrates present were fermented. Old cultures gave a powdery, grit-like precipitate when precipitated with beta-naphthylamine.

One of the typical reactions of wood decay fungi on artificial agar media is the production of brown zones on malt extract media to which tannic acid has been added. This is the standard medium for the separation of white-rot from brown-rot fungi. Techniques and results of studies on wood decay fungi on this medium are given by Davidson, Campbell, and Blaisdell (1938); Davidson, Campbell, and Vaughn (1942), and Nobles (1948). Ledingham and Adams (1942) tested 29 white-rot fungi for their ability to decompose the lignin of calcium lignosulphonate (considered to be a more accurate medium than spent sulphite liquor) over a period of 30 days in stationary liquid culture. Species giving a negative or weak tannic acid medium reaction decomposed no or only small amounts of lignin, while 10 of the 13 of those giving a strong reaction decomposed 6.9 to 14.2% of the lignin in 30 days. Cultures of Alternaria and Fusarium used most of the sugar and 8.3 to 18.3% of the lignin in such solutions after 40 days. Soil fungi used included 26 species in 12 genera. Most of these fermented most if not all the sugars present, and lignin

decomposition varied from 0.3 to 12.3%. Thirteen strains of 8 species of Fusarium decomposed 4.4 to 13.4% of the lignin content of samples in 20 days, and 33 strains of Alternaria species decomposed 2.8 to 18.2% of the lignin in 20 days.

Adams and Ledingham (1942b) on comparing beta-naphthylamine precipitation and ultraviolet spectrographic methods of measuring sodium lignosulphonate in culture media found the latter technique the more accurate.

Bollen (1942, 1955) has found that ammonia-base spent sulphite liquor can be applied to alkaline soils with good results since the ammonia and sulphur are readily available to the microbial population and the lignin may contribute to the soil humus. No increase in numbers or kinds of organisms was noted, and pH of the soil apparently is not changed.

Strains of Aspergillus, Penicillium and Rhizopus were reported by Enkvist and Backlund (1955) to be able to grow in spent sulphite liquor when 0.04% NaNO_3 and 0.02% KH_2PO_4 were added. White-rot fungi required added carbohydrates.

Kleinert and Joyce (1955) found that after three processings of lignosulphonic acid supernatants from short or long cooks with sodium or calcium bases, certain molds of several genera including Aspergillus and Penicillium were able to survive in aerobic culture. It was thought that most if not all carbohydrates had been removed and that these molds were growing on the lignosulphonic acid present.

Use of fungi in chemical studies on lignin. To obtain lignin in a purer state than by using available chemical techniques, Schwalbe and Eckenstam (1927) exposed pieces of pine wood to the action of Merulius lacrymans, the dry-rot fungus. On the basis of the product they obtained, which was more rapidly and completely freed of cellulose, as well as more easily filtered, it was concluded that lignin acids were formed by the fungus.

Four common brown-rot fungi have been used in experiments in Sweden dealing with the lignin content of decayed wood. In most cases (Apenitis, Erdtman and Leopold, 1951) it was found that after most carbohydrates had been used up, these fungi will attack the lignin residues. Values for each species were sometimes erratic or difficult to reproduce. Results of such experiments are of importance since most of the carbohydrates can be removed in this way and the altered lignin may be more readily available for byproduct synthesis.

When new wood samples and samples decayed by brown-rot fungi were compared (Leopold, 1951) it was found that almost complete delignification can be effected by sulphonation rather rapidly in neutral media. Lignin product yields decrease with increasing age of decay. Ethanolbenzene and alkali extractives give higher vanillin yields than the remainder of the lignin. Based on such work, Leopold (1952) obtained better yields of vanillin from decayed waste wood than from nitrobenzene oxidation of lignin waste products.

Nord and his students, (Kudzin, de Baum and Nord, 1951; Kudzin and Nord, 1951; Nord and Schubert, 1949; Nord and de Stevens, 1951; and Schubert and Nord, 1950a, 1950b, 1955) have studied problems associated with lignin

chemistry both from the point of view of lignins isolated chemically and isolated enzymatically. To obtain lignins of the latter type, several species of brown-rot fungi have been used. With such materials it is hoped that problems relating to formation and function as well as chemistry of lignin can be resolved. Larger amounts of chemically similar lignins are liberated through action of fungus enzymes than with chemical techniques.

Gottlieb and Pelczar (1951) have presented a review of the general work done up to 1951 on lignin degradation microorganisms, and they summarized the information available on the chemistry of lignin. In 1953, Day, Gottlieb and Pelczar (1953) showed that while P. versicolor could utilize lignin in a natural state in wood and other preparations, it could remove sodium lignosulphonate from solution but not utilize it as a sole carbon source. They concluded that rather than utilize the lignin-containing molecules these were only caused to adhere to the outer wall of the mycelium, thus removing them from solution, rather than causing their degradation.

In studies designed to differentiate between brown-rot and white-rot fungi, Davidson, Campbell and Blaisdell (1938), and Davidson, Campbell and Vaughn (1942), followed by a comprehensive treatment by Nobles (1948), developed techniques for study and cultural identification of such fungi. The work of Perlman (1949) on one species, of Yusef (1953) on vitamin requirements, and of Jennison et al. (1955) on nitrogen requirements of some of these fungi in submerged culture in synthetic media, has paved the way for the development of techniques for study of the potential use of lignin in pure culture at least by the white-rot Basidiomycetes.

Conclusions

Many wood-decay, some litter-decomposing, and a few soil fungi have been shown to be able to utilize lignins in varying forms. Very little work has been done on the ability of these and other fungi to utilize the lignins in waste products of the paper and fiber industries either in pure culture, mixed culture, or in enriched cultures using nutrient supplements which such fungi require but which would not normally be present in wood and in materials added to the processes of making wood fibers available to varied industrial uses. It is possible that in this reservoir of virtually untapped resources there may be organisms able to, or which may be induced to, utilize some or all of the materials in waste liquors and at the same time produce tissues useful or more readily disposable.

B. Some Effects of Spray Disposal of Spent Sulphite Liquor on Soil Mold Populations *

Introduction

The use of additives for the improvement of soils is as old as our knowledge of agricultural pursuits. Early farmers knew that if crop residues were returned to the soil the succeeding crop was improved. The deposit of an annual increment of silt on the floodplain of the Nile was welcomed by the early Egyptian farmer because it gave his next crop a stimulus for growth. The addition of barnyard manure to the soil of a field planted to one crop year after year helped maintain the yield of that crop. It has been thought that as a means of disposal a similar use might be made of other wastes, such as spent sulphite liquor.

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Within the last 50 years we have found that the principal reason for the increased activity in soils following the application of barnyard manures and the plowing under of last year's stubble or this year's green cover or green manure crop, is related to the activity of countless numbers of microorganisms. Some bacteria and fungi will attack the sugars which are released by mechanical break down on the plant tissues, others will attack the cutin on the leaf or stem surface, others will attack constituents of protoplasm, others will attack the cellulose, and finally a few will attack the lignin. The net result of such attacks will be the reduction of these applied or plowed-in residues, the release of complex carbonaceous, nitrogenous and other compounds, their reduction to simpler compounds, mineralization, and greater availability for use by the next crop plant to be applied to the particular field.

In a somewhat different way, yet not too dissimilar, the microorganisms of an area will react on any material applied to a particular soil. Thus, when spent sulphite liquor is applied to a soil, given optimum conditions of growth for the population of microorganisms which may be present, those substances which are present in the liquor and which are available to the organisms will be attacked, reduced, made usable by other organisms, and a treatment process will be effected.

Background of problem and area of study

At the 13th Purdue Industrial Waste Conference, R. M. Billings (1959a) described the method of disposal of spent sulphite liquor in excess of that which could be safely absorbed by the Menominee River at Niagra, Wisconsin. Briefly, the technique was to pipe the spent liquor

across the river to the Michigan side where it was stored in a stainless steel tank until hauled away in a stainless steel tank truck to a 40-acre field 1.5 miles distant where, through a special piece of equipment, it was atomized and sprayed onto the soil at the rate of one pound of solids per square yard per 24-hr period. Amounts of spent sulphite liquor disposed of in this way have been as follows:

1956	2,000,000 gal.
1957	15,000,000 gal.
1958	8,000 gal.
1959	0 gal.

Following presentation of his report it was suggested that it would be of interest to discover to what extent this type of land use or soil additive affected the fungus populations of the soil, and, possibly, conversely, what effect these populations might have had on the spent sulphite liquor. Mr. Billings and his organization very kindly furnished samples of soils composited from several test holes drilled with a post auger. Upon finding that the samples were only from soils to which spent sulphite liquor had been applied, an additional request was made for two shallow samples, one from untreated soil nearby, the other from treated soils. All these samples were obtained at the end of the treatment season in the autumn of 1958. In June, 1959, another series of samples was obtained. This time the samples were taken from one 15-foot and one 20-foot auger drilled hole in a treated area, and similar holes in an adjacent untreated area. Data to be presented in this section were obtained from these three sets of samples. Figures 46, 47, and 48 are based only on the June, 1959, samples.

FIGURE 45
ORGANIC MATTER IN TREATED AND UNTREATED SOILS

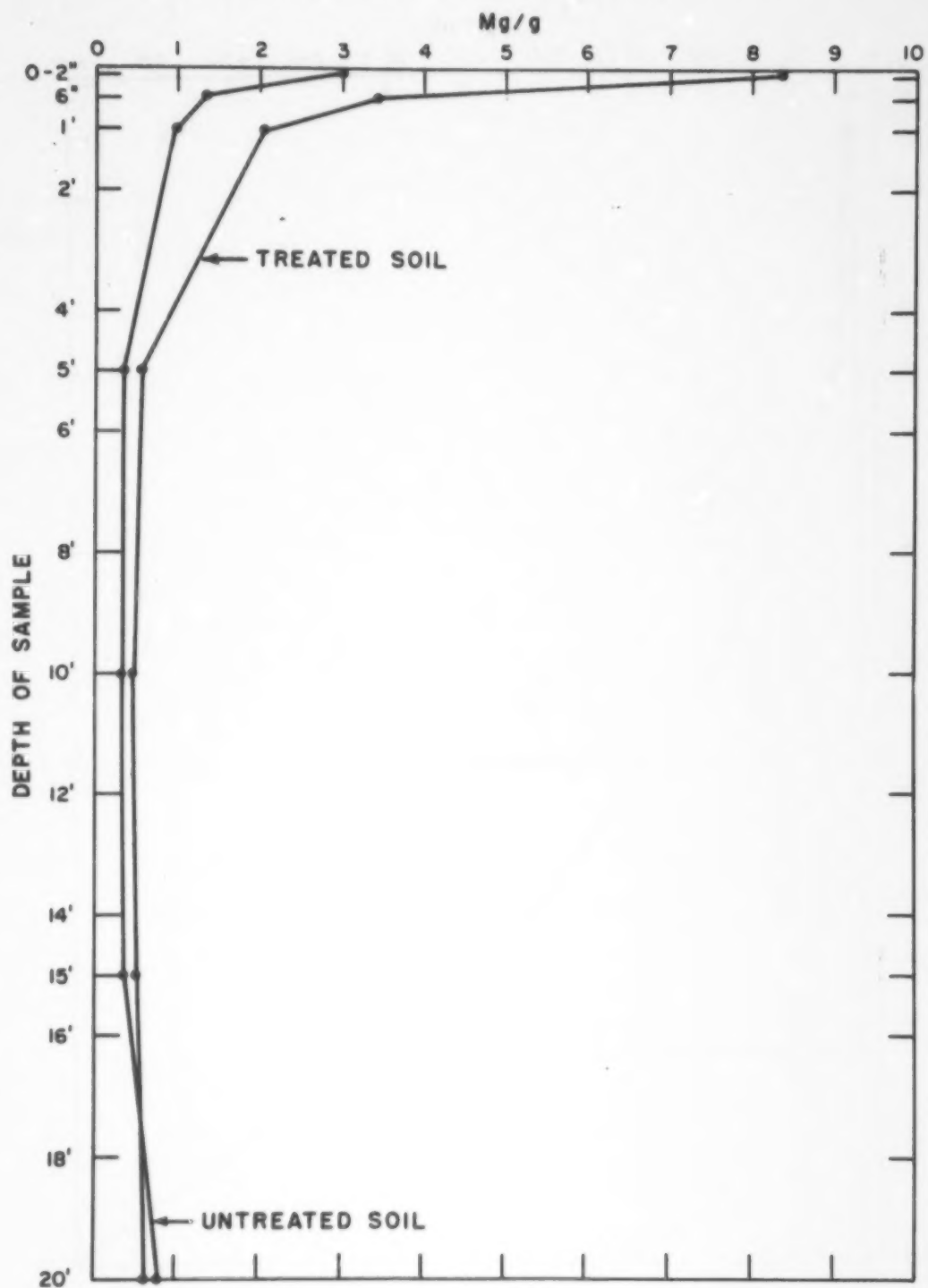


FIGURE 47
FOLIN-DENIS REACTING MATERIALS
IN TREATED AND UNTREATED SOILS

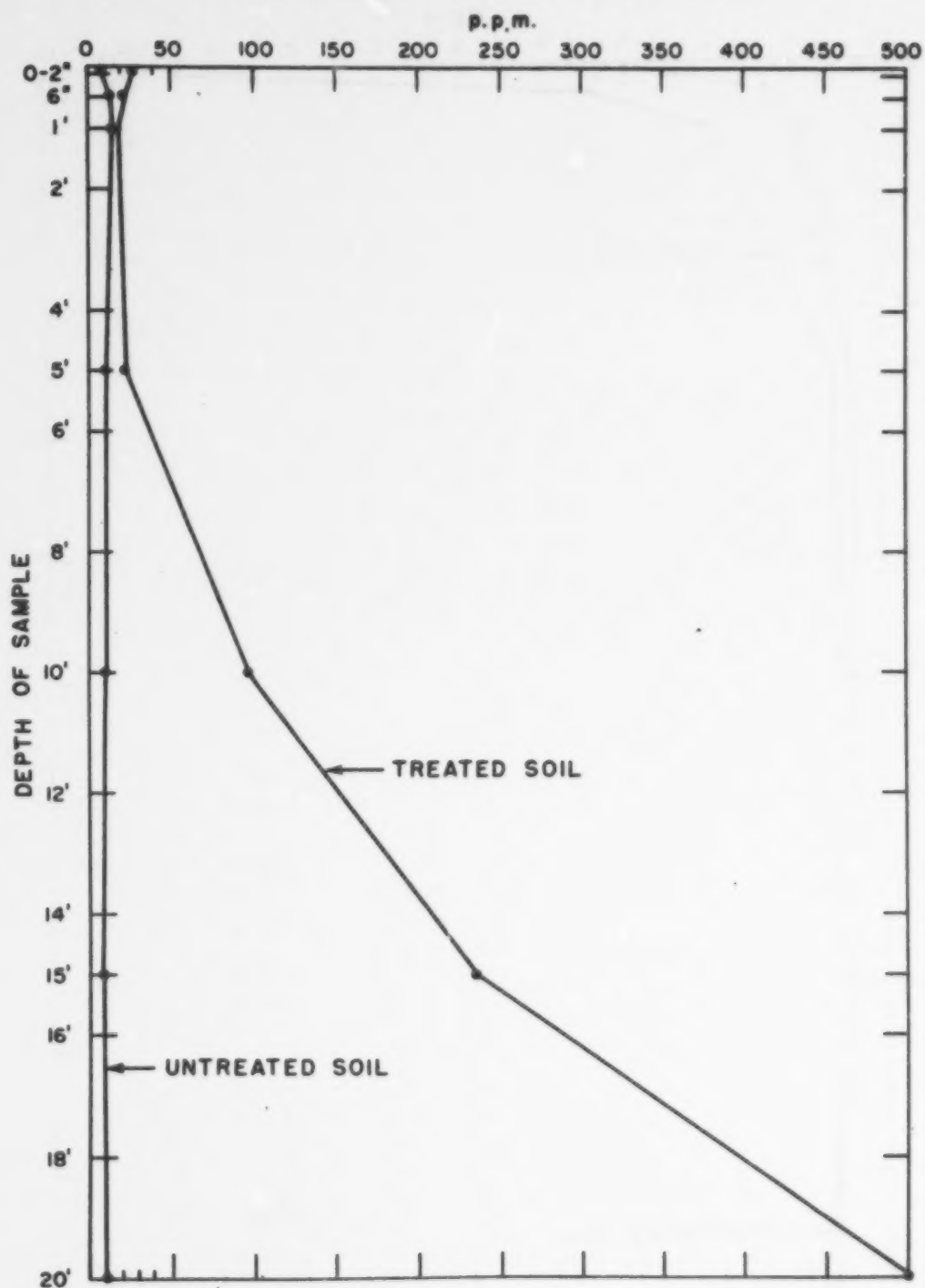
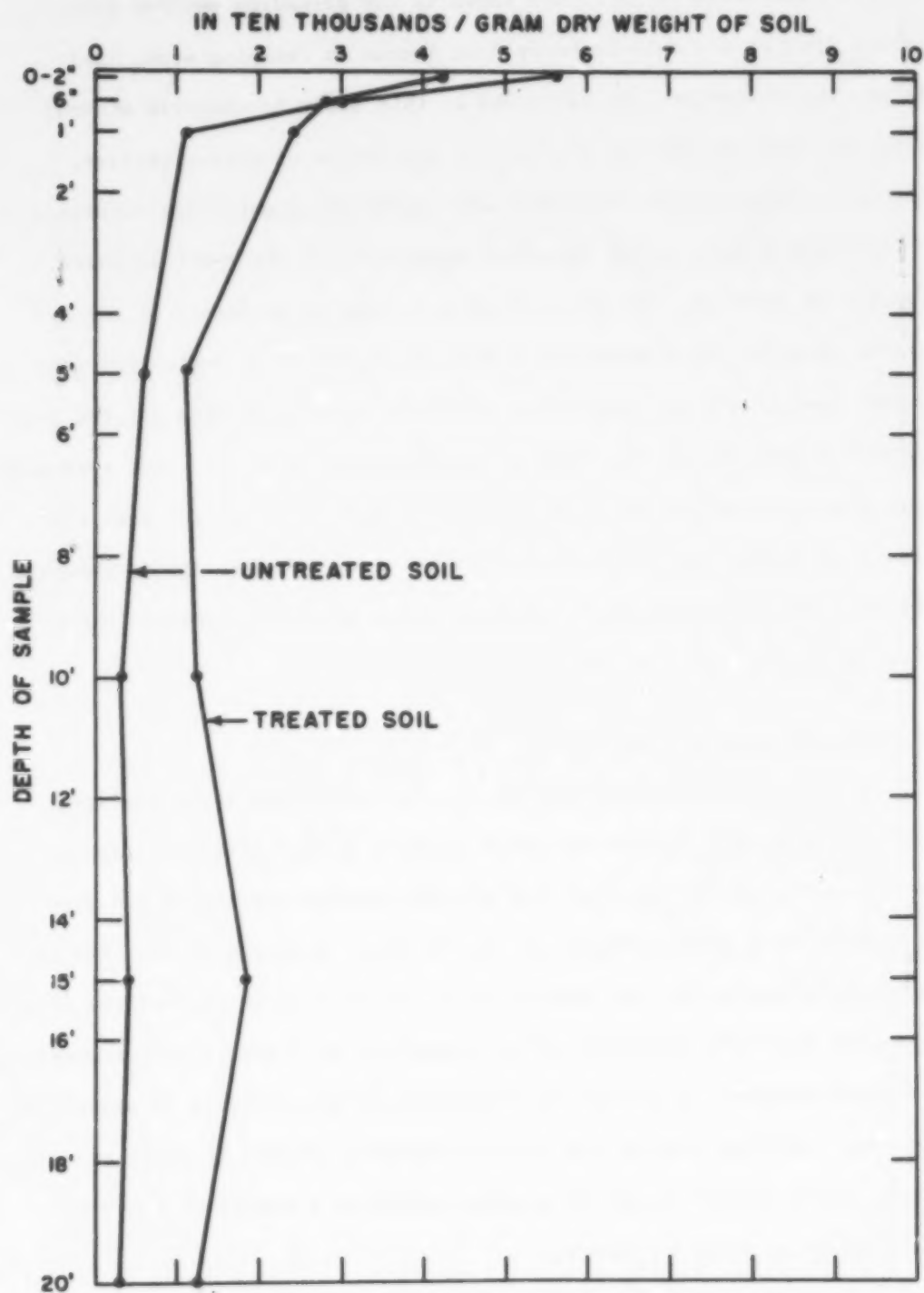


FIGURE 48
COLONIES OF FUNGI IN TREATED AND UNTREATED SOILS



In work with fungi it was shown in the preceding section that certain species are able to break down lignin in decaying wood, leaf litter, and elsewhere. It was hoped in this study to discover whether or not any natural members of the soil population of microorganisms, especially fungi, could break down and remove the lignin-like materials in the lignosulfonic acids and other substances in the spent sulphite liquor. In addition, the soils which were used as receptors of the sprayed materials were notoriously poor in quality. In fact, they were so poor that it was not considered practical to utilize them for the production of tree farms, let alone any agricultural crop. In some instances waste liquors from the paper pulp industry have been used for soil additives in programs designed to improve agricultural soils. It is possible that with the continuing application of spent sulphite liquor to these soils some improvement in quality may result.

The soils and natural vegetation

According to Billings (1959a, b), the soils onto which the spent sulphite liquor was sprayed are sands lying in a deep preglacial trough. The sand accumulation resulted from glacial moraine deposition and from the deposit of a large quantity of coarse sand. Drainage of the area is apparently adequate for the removal of all or of a large part of the silts, clays, and colloidal materials which accumulate as a result of decomposition of mineral residues of glacial materials and of the activity of plant life which both utilizes some of the mineral elements present in soils and adds to the soil a certain amount of organic matter as a result of death of whole plants or parts of plants.

The area under consideration lies in Dickinson County, Michigan, almost directly across the Menominee River from Niagara, Marinette County, Wisconsin. By inference from chapters in "The Vegetation of Wisconsin" by Curtis (1959), we can assume that the plant community involved is a xeric pine barren. It is xeric because of rapid percolation of water through coarse sand, rather than because of low precipitation. Because of rapid percolation of water only those plants adapted to live so that they can make the best use of the available water when it is available can survive. Because of this, any materials added to the soil which will tend to aid in retention of moisture are useful adjuncts to the improvement of a plant community provided the additives contain no toxic substances, that the total additives which may come in contact with the plants involved do not create a toxic situation, or that the additives do not eventually build up an impervious layer.

No data are available on the composition of the plant cover or microbiology of the spray area before spraying was started. However, such information can be gained from adjoining areas of comparable nature. Data which have been obtained on the soils will be presented below as comparisons between soils of an adjoining nonsprayed site, and sites within the spray area.

On the basis of samples received at Cincinnati, certain information has been obtained. This includes the pH of soil suspensions, percent of water lost at 105°C, organic matter lost on flashing in the 600°C oven, and the ppm of materials tested by means of the Folin-Denis reagent test as read on the Beckman Model DU spectrophotometer under ultraviolet light

at the wave length .282. While this wave length usually reports, on the basis of the Folin-Denis reagent, any material phenolic in nature, it is used in some instances as a "finger-print" of lignin. This is understandable because lignin is phenolic as are the similarly resistant tannic acid and vanillin which are also identified by the use of the .282 wave length.

Methods

The data reported concerning the soils tested were obtained by the use of several relatively standard techniques for describing soils. Soil samples were collected by means of an auger at depths of 2 in., 6 in., 1, 5, 10, 15 and 20 ft. It was thought at the time of sampling that 20 ft. was deep enough to penetrate into the sample sites. Composite samples from 4 borings were made up for depths from 1 ft. down to 20 ft. in September 1958 at the end of the spray season. In October, 1958, composite samples from several points in sprayed and unsprayed areas were collected at the shallower depths. In June, 1959, after about 1.5 years of relative inactivity, paired samples from each depth in a 15 ft. and a 20 ft. core were obtained.

Samples were taken wet, placed aseptically in Mason preserve jars, packed in a wooden box for safe travel, and shipped to Cincinnati. At the laboratory, 1:10 dilutions of each sample were prepared as described earlier, and from these pour plates in neopeptone-dextrose-rose bengal-Aureomycin agar were prepared. For yeast isolation 1:10 dilutions of the samples were used in YNB-1% and YNB-20% glucose solutions in shaken culture.

Using the supernatant from the 1:10 dilution of the soil sample, the pH was determined with a glass electrode pH meter (Table 66).

Paired samples of the same soils were placed in tared metal soil cans. Wet weights were obtained (Table 66), the soils were dried at 105°C and weighed again, and finally the samples were transferred to porcelain crucibles and flashed at 600°C. The percentages of free water lost from the samples are reported in Table 66, and the amount of organic matter lost is given in mg/g in Figure 46.

To determine the amount of lignin or lignin-like materials in each sample of soil, 100 gm lots of soil (wet weight) were removed from the glass bottles which had been stored in the 5°C constant temperature chamber, to 250 ml. Erlenmeyer flasks. To these were added 100 ml distilled water. During each of two days these flasks were hand shaken several times and allowed to stand overnight. After a final hand shaking the supernatant was filtered off through Whatman No. 1 filter paper and 50 ml retained. Twenty-five ml of this supernatant were tested with Folin-Denis reagent. Where large amounts of test materials were present, this quantity was reduced to 5 ml and in two cases to 1 ml. All samples were corrected to give readings of ppm Folin-Denis testing materials (Fig. 47) by using a standard curve based on a sample of waste Kraft liquor which was available. For comparison with the spent sulphite liquor, a comparison of the five samples using both comparison techniques showed that the waste Kraft liquor was about 80 times as strong as the spent sulphite liquor and the proper corrections were made.

Table 66. Characteristics of soil samples.

Sample Date	Depth	pH		Wet Weight, 15 ml		Water loss, %		Phenolic compounds, ppm	
		*unspr.	spr.	unspr.	spr.	unspr.	spr.	unspr.	spr.
Oct. 1958	0-2 in.	6.2	5.8					19	36
	6 in.	6.5	6.0					31	56
	1 ft.		5.8						35
	5 ft.		6.1						30
	10 ft.		6.8						188
Sept. 1958	15 ft.		6.9						18
	20 ft.		7.0						29
June 1959	0-2 in.	6.1	5.6	32.6	30.3	9.7	17.5	8	42.5
	6 in.	6.3	4.7	37.1	32.0	5.5	11.8	7	22.5
	1 ft.	6.4	5.1	40.5	40.7	4.2	6.7	14	17.5
	5 ft.	6.9	6.7	40.1	41.3	3.9	6.3	13.5	26
	10 ft.	7.2	6.4	41.8	40.3	6.5	5.2	10.5	109
June 1959	15 ft.	7.4	6.7	40.2	41.4	4.6	3.7	7	76
	0-2 in.	6.2	5.5	36.1	30.1	8.7	22.1	8	125
	6 in.	6.0	5.5	38.7	37.4	7.6	12.7	19	19.5
	1 ft.	5.8	5.5	40.6	42.6	6.7	9.6	15	19
	5 ft.	6.3	6.0	40.8	44.2	5.1	4.8	5.5	16
June 1959	10 ft.	6.5	6.3	37.2	36.9	2.9	11.5	7.6	81
	15 ft.	6.9	7.1	37.5	38.7	3.3	3.4	7	382
	20 ft.	7.5	7.3	45.8	42.6	2.2	9.6	6.5	500

* unspr. - unsprayed, spr. - sprayed.

In addition, a mechanical analysis was made to determine the relative amounts of sand, silt, and clay in the samples. The remainder of the soil sample was air dried, passed through a 200 ml sieve, 100 g lumps were removed, 5 ml of sodium oxalate were added, then the whole was blended in a motor dispersion unit for 15 min to disperse all clumped soil particles. The soil was then transferred to a 1000 ml cylinder, the volume was brought to 1000 ml with distilled water and shaken by hand by inversion and reversion for 1 min. A hydrometer was inserted at the completion of shaking, and at the end of 40 sec and 2 hr was read. Temperatures were read at the same time since the hydrometers were calibrated at 67°F, and the reading was corrected.

Results

Results of the wet weight, water content, and pH determinations are presented in Table 66. Organic matter determinations are presented in Figure 46 and of Folin-Denis reagent reactive materials in Figure 47. Results of mechanical analyses of soils are listed in Table 67.

Number of organisms isolated from the several soil samples are listed in Table 68 and numbers of fungus colonies found are presented in Table 69 and Figure 48. To the extent that species or species groups have been determined, reports on numbers of colonies of fungus species isolated from unsprayed soils are given in Table 70, and those from sprayed soils in Table 71. In the latter two tables the parenthetical figure following the number of colonies indicates the number of samples from each location in which the fungus was found. The average total number of colonies is not the true total of the column at the bottom of which it

Table 67. Mechanical analyses of soil samples

Date	Sample Depth	*Unsprayed Sprayed	Separates		Texture
			% sand	% silt	
Sept. 1958	1'	S	96.6	0.7	2.7
	5'	S	96.8	0.7	2.5
	10'	S	97.2	0.1	2.5
	15'	S	97.3	0.0	2.9
	20'	S	97.1	0.3	2.6
June 1959	0-2"	U	96.1	0.7	3.2
	6"	U	96.0	1.1	2.9
	1'	U	96.5	0.5	3.0
	5'	U	96.5	0.0	3.8
	10'	U	94.2	1.7	4.1
June 1959	15'	U	96.1	0.6	3.3
	0-2"	S	94.7	1.3	4.0
	6"	S	95.2	1.0	3.8
	1'	S	95.1	1.5	3.4
	5'	S	95.8	1.3	2.9
June 1959	10'	S	96.3	0.9	2.8
	15'	S	96.2	1.0	2.8
	0-2"	U	94.9	1.4	3.7
	6"	U	94.9	1.5	3.6
	1'	U	95.2	1.3	3.5
June 1959	5'	U	96.7	0.2	3.1
	10'	U	97.0	0.0	3.0
	15'	U	96.5	0.7	2.8
	20'	U	94.7	1.6	3.7
	0-2"	S	93.9	2.1	4.0
June 1959	6"	S	94.2	1.9	3.9
	1'	S	95.3	1.0	3.7
	5'	S	96.6	0.4	3.0
	10'	S	96.2	0.8	3.0
	15'	S	97.0	0.2	2.8
June 1959	20'	S	96.1	1.0	2.9

Table 68. Bacteria, Actinomycetes, and Fungi in Soils.

Sample date	Depth	Bacteria per g. at 1:500,000 in 100,000's		Actinomycetes per g. at 1:10,000 in 1000's		Fungi per g at 1:10,000 in 1000's	
		Unspr*	Spr	Unspr	Spr	Unspr	Spr
June 1959 15 ft. core	0-2 in	34	100	51		5.0	44
	6 in	14	89	14	3.6	3.1	28
	1 ft	39	66	13	0.7	3.1	12
	5 ft	39	65	13	0.5	1.4	5.4
	10 ft	52	65	6.5	0.5	1.7	2.1
	15 ft	55	63	5.2	0.4	3.0	2
June 1959 20 ft. core	0-2 in	76	105	21		3.3	85
	6 in	70	67	34	4.6	2.5	43
	1 ft	66	65	21	5.2	1.6	17
	5 ft	65	60	2.6	2.4	0.9	5.8
	10 ft	70	55	2.8	3.1	0.8	1.7
	15 ft	70	135	2.8	1.4	0.6	4.3
	20 ft	55	170	11	1.7	1.2	4.6

Table 69. Percent Occurrence of Fungi in Soils.

Total colonies observed (as total of average totals for each depth.)	Unsprayed	Sprayed
Per cent in 0-2 in level		
6 in	12,953	94,610
1 ft	24.9	47.0
5 ft	17.2	25.4
10 ft	18.3	13.6
15 ft	8.8	2.7
20 ft	7.3	2.7
	13.8	3.5
	9.6	5.2

Fungus	Sample Depth						
	0-2 in	6 in	1 ft	5 ft	10 ft	15 ft	20 ft
Average total colonies	3232 (3)	2229 (3)	2372 (2)	1140 (2)	950 (2)	1790 (2)	1240 (1)

Fungus	Sample Depth						
	0-2 in	6 in	1 ft	5 ft	10 ft	15 ft	20 ft
Average total colonies	3232 (3)	2229 (3)	2372 (2)	1140 (2)	950 (2)	1790 (2)	1240 (1)

Species present only in unsprayed areas

Species present in both unsprayed and sprayed areas

Pyof

Table 71. Percentage average Total Colonies of Fungi in a Sprayed Area.

Fungus	Sample Depth					
	0-2 in	6 in	1 ft	5 ft	10 ft	20 ft
Average Total Colonies	44467 (3)	24043 (3)	12825 (3)	2535 (3)	2550 (3)	4880 (2)
Species present only in sprayed areas						
<i>Cladosporium cladosporioides</i>		0.03				
Dematiaceae spp.			0.82			
<i>Aspergillus niger</i>					16.6	
<i>Fusarium aquaeductum</i>			2.0		2.4	
<i>Trichosporon</i> spp.	0.77	2.5	0.41			1.4
<i>Rhizopus</i> sp.	1.4					
<i>Sporotrichum</i> sp.	0.19					
	0.96		0.41		1.5	2.9
Species present in both sprayed and unsprayed areas						
<i>Phoma</i> sp.	0.07	2.6				
<i>Zygorynchus moelleri</i>	0.04	0.03	0.83			
<i>Mucor</i> spp.	0.11	0.3	2.7			
<i>Phialophora jeanselmei</i>	0.04	0.46		16.7	14.1	10.0
Moniliaceae spp.	3.0	3.3	2.2	4.3	2.0	
<i>Penicillium</i> spp.	0.73	3.8	4.1	1.3	11.8	
<i>Penicillium sclerotiorum</i>	6.7	0.6	2.5			
<i>Penicillium lilacinum</i>	0.87	1.7	2.9	3.7	5.5	12.5
<i>Aureobasidium pullulans</i>	0.91	2.0	10.2	34.4	18.0	20.9
<i>Trichoderma viride</i>	0.42	1.3	1.1	1.1	2.0	1.4
<i>Paecilomyces varioti</i>		0.03				
<i>Candida</i> spp.	92.3	87.9	41.8	13.6	10.2	25.2
<i>Cryptococcus</i> spp.	31.5	35.9	28.7	22.8	6.2	7.0
<i>Rhodotorula glutinis</i> ss. lat.	0.18		0.41	3.9	3.9	1.4
<i>Aspergillus</i> sp.	0.93	1.5	1.2			
Moniliaceae sp. (Fat producer)	3.8	1.2	1.6			
<i>Gonytrichum caesium</i>	5.0	2.5	0.41			1.4

stands. It is presented to compare the results of each separate type of test. In a supplemental section of Tables 70 and 71 (Table 72), yeasts are listed as present or not because in the isolation technique used there is no way to quantitate the results. These yeast names are specific identifications of generalized generic categories listed in the upper parts of the tables.

Table 72 gives data concerning numbers of yeast cells observed based only on the original sample diluted to 1:10. These are minimum values except for Rhodotorula mucilaginosa from the unsprayed area. Of those isolated on pour-plates, it is the only yeast that could be identified as to species and correlated with the habitat. The other yeast isolates from pour-plates represent groups of species which had to be purified before identification was possible, and on this basis enumeration is not practical. In Table 72, where 20 cells are indicated, the species were isolated from both 1 and 20% glucose flasks; where 10 cells are indicated, isolation was made from either one or the other medium.

In the identification of yeasts it is important to know some of the carbon-containing compounds available to the cells of a colony as carbon sources. The replicator technique for determining availability of various carbon sources to yeast strains in agar culture was used to obtain the data presented in Table 73. Eight of the commoner species are listed, and the results are based on the growth capabilities of one or more strains of each of these species from this particular set of isolations. Carbon-use patterns are not different for most strains isolated from other habitats.

Table 72. Composite list of Yeasts.

Species	Depth of sample (composite of a 15 ft and a 20 ft core)					
	0-2 in	6 in	1 ft	5 ft	10 ft	15 ft
Unsprayed area						
<i>Candida krusei</i>				10		
<i>Cryptococcus albidus</i>						10
<i>Cryptococcus laurentii</i>				10	20	
<i>Rhodotorula glutinis</i>			10	10	10	20
<i>Rhodotorula mucilaginosa</i>			1000			10
<i>Torulopsis farnata</i>			10	10	20	10
Sprayed area						
<i>Candida</i> sp.						
<i>Candida guilliermondii</i>						10
<i>Candida krusei</i>		20	10			
<i>Candida lactis-condensii</i> (nr.)			20	20	20	20
<i>Candida melinii</i>				10		
<i>Candida pelliculosa</i>			20			
<i>Cryptococcus albidus</i>	10					
<i>Cryptococcus laurentii</i>	10	10	10		20	20
<i>Rhodotorula glutinis</i>					20	20
<i>Sporidiobolus johnsonii</i>				10		
<i>Torulopsis farnata</i>	20					
<i>Torulopsis conspicua</i>			10		10	
<i>Torulopsis stellata</i>			10			

Table 73. Utilization of 34 carbon sources by 8 species of yeasts.

Carbon Source	Yeast Species							
	1	2	3	4	5	6	7	8
Glucose	+++	+++	+++	+++	+++	+++	+++	+++
Galactose	+	+++	++	+++	+++	+++	+++	+++
L-sorbose	-	+++	++	++	-	-	+	+
Maltose	T	+++	+++	+++	+++	+++	+++	+++
Sucrose	+	+++	+++	+++	+++	+++	+++	+++
Cellobiose	-	+++	+	+++	+++	+++	+++	+++
Trehalose	-	+++	+	+++	+++	+++	+++	+++
Lactose	-	T	-	-	+++	+++	+++	+++
Melibiose	T	+++	-	++	+++	+++	-	-
Raffinose	T	+++	T	+++	+++	+++	+++	+++
Melezitose	-	+++	+++	+++	+++	+++	+++	+++
Inulin	-	+++	+++	+++	+++	+++	+++	+++
Soluble Starch	-	+++	T	+	T	T	T	-
D-Xylose	+	+	T	-	+	+	-	-
L-arabinose	-	T	-	+++	+++	+++	+++	+++
D-arabinose	-	+	-	+++	+++	+++	+++	+++
D-ribose	-	-	-	+	+	+	+	+
L-rhamnose	-	-	T	T	+	+	+	+
Ethanol	+++	++	+++	+	+++	+++	+	+
Glycerol	-	-	+++	+++	-	-	+	+
D-erythritol	-	-	+++	+++	+	+	+	+
Adonitol	-	T	+	-	+++	+++	+	-
Dulcitol	-	-	+	+++	+++	+++	+++	+++
D-mannitol	-	++	+	+++	+++	+++	+++	+++
D-sorbitol	-	+++	+	+++	+++	+++	+++	+++
alpha-methyl-D-glucoside	-	+++	-	+++	+	T	T	T
Salicin	T	T	+	+++	+++	+++	+++	+++
K-gluconate	-	-	+	+	+++	+	+++	+++
Ca-2-keto-gluconate	-	+	-	-	+++	+++	+++	+
K-5-keto-gluconate	-	+	-	+++	+++	+++	-	-
DL-lactic acid	++	T	+	-	+	+	-	-
Succinic acid	++	++	+++	+++	+	T	++	-
Citric acid	-	T	+++	+++	+	+	+++	+++
D-Inositol	-	-	+	+	+	+	+++	+++

Species: 1. Candida krusei (1 strain tested)
2. Candida guilliermondii (1 strain)
3. Candida pelliculosa (1 strain)
4. Torulopsis famata (6 strains)

5. Cryptococcus albidus (2 strains)
6. Cryptococcus laurentii (6 strains)
7. Rhodotorula glutinis (3 strains)
8. Rhodotorula mucilaginosa (1 strain)

One way to express similarities or differences between two or more habitats is through the use of a coefficient of similarity (Christensen, et al, 1963). This coefficient is obtained by the formula $2w / a + b$, in which w is the number of species common to two areas, a the number of species found in one of the two areas, and b the number of species found in the other area. Coefficients of similarity are given in Table 74 for yeast populations, populations of filamentous fungi, and the whole fungus population of the sampled soils.

Discussion

A sandy soil upon which spent sulphite liquor has been sprayed has become, after three years' treatment, more acid near the surface, has a richer organic matter content in surface and subsurface layers, and, depending on the area in the field, has allowed percolation of lignin-like materials to depths of 10, 15 or 20 ft where they may be retained for a considerable period of time, or, apparently, may be carried further down through the profile of this coarse grained soil.

These facts were discovered in several ways. The hydrogen ion concentration is higher near the surface levels, decreasing downward. Nonsprayed soils are generally less acid in the upper layers, becoming more alkaline down to 20 ft. Acidity at all levels in sprayed soils is higher than in unsprayed soils.

All soil samples tested by mechanical analysis (Table 67) proved to be sandy in texture. In the 31 samples, the range of clay content was 2.5 - 4.1 percent, silt content 0 - 2.1 percent, and sand 93.9 - 97.3 percent. In all cases, on the textural triangle, these values lead

Table 74. Coefficient of Similarity.

Population	Numbers of Species		Found in Sprayed area (b)	Coefficient of Similarity $2\sqrt{a \div b \times 100}$
	Common to both areas (w)	Found in unsprayed areas (a)		
Yeasts	5	6	13	52.6
Filamentous Fungi	14	19	31	70.0
Yeasts and filamentous fungi	19	25	34	65.5

only to a conclusion that the sample is sand. Thus, the three years' application of spent sulphite liquor and attendant microbiological activity have had little effect on the basic sandy texture of these soils.

Water is held more tenaciously in the upper layers of sprayed than unsprayed soil in almost direct ratio to the amount of organic matter which is present. Organic matter is present to a greater extent in upper layers of this soil than lower, that present in lower levels in either instance being insignificant as a basis for differentiating the two types of sample. However, materials reacting strongly with the Folin-Denis tyrosine reagent occur much more abundantly on the surface and at lower levels in sprayed sites than in unsprayed sites. The amount of such materials, in favorable habitats in the same field, increases to a considerable extent over that present in the upper levels in the soil.

The effects of the organic matter and some of its fractions mentioned above are noticeable on the populations of microorganisms assumed to be present in the soil on the basis of isolations which have been made to date. In all cases plate counts were made on the basis of expected fungus populations. Colony counts of bacterial colonies are usually made on much more dilute samples, so the numbers of colonies reported in Table 68 are not critical but only indicate trends and effects on populations at the dilutions and on the media used for fungus studies. The same is true for reports of Actinomycetes observations.

On the basis of media used, dilutions of samples from which plates were poured, and counts which were obtained, it is apparent that much smaller numbers of mold colonies occurred at lower levels and on unsprayed

soils than at higher levels in the soil and on sprayed soils. A relatively high population of molds occurred in deeper layers of the soil. This could be due to either the gradual washing of spores downward in soils as reported by Burges (1953, 1958) thus building up a large soil spora, or it could result from growth in available nutrients.

That the latter effect is involved is evident from the larger concentration of disseminules in sprayed than in unsprayed soils at depths of 15 and 20 ft, especially in respect to yeast populations. The level of least numbers of spores does not necessarily correspond to the level of maximum accumulation of Folin-Denis reagent reactive materials. However, in all cases, at about the 10 ft level, there is a build-up of such materials and a decrease in number of mold colonies. Below this level such colonies tend to increase in number indicating a greater availability of nutrients accompanying the spray materials.

It may be said that in general there is a gradual decrease in numbers of bacterial colonies downward. However, in one instance this is not the case, and in another the pattern of numbers of colonies matches that of the fungi in that at 10 ft there is an inhibitory effect with greater numbers of colonies downward. Following the spraying of spent sulphite liquor on the soil there is a very pronounced effect on the Actinomycetes which are definitely inhibited on sprayed soil.

Sixty species of fungi, including yeasts, were recovered (Table 75) by various techniques from soils to which spent sulphite liquor had been added in atomized form. In an adjacent area, only 55 species were recovered (Table 75). It is shown that the addition to soil of spent sulphite liquor and its accompanying BOD-producing impurities in the waste water

Table 75. Fungi observed in isolations from soils receiving spent sulphite liquor.

Species	Observation Number		
	Total	Untreated	Treated
Moniliaceae	109	46	63
<u>Penicillium</u> spp.	84	32	52
<u>Trichoderma</u> <u>viride</u>	63	18	45
<u>Phialophora</u> <u>jeanselmei</u>	60	28	32
<u>Aureobasidium</u> <u>pullulans</u>	57	22	35
<u>Penicillium</u> <u>lilacinum</u>	56	24	32
<u>Mucor</u> spp.	30	14	16
<u>Penicillium</u> <u>janthinellum</u>	23	7	16
<u>Candida</u> spp.	19	2	17
<u>Cladosporium</u> <u>cladosporioides</u>	16	9	7
<u>Cryptococcus</u> spp.	16	1	15
<u>Fusarium</u> <u>oxysporum</u>	13	7	16
<u>Penicillium</u> <u>funiculosum</u>	13	4	9
<u>Rhodotorula</u> spp.	13	4	9
<u>Zygorhynchus</u> <u>moelleri</u>	13	4	9
<u>Phoma</u> spp.	13	8	5
<u>Aspergillus</u> <u>flavus</u>	11	5	8
<u>Fusarium</u> <u>aqueductum</u>	11	3	8
<u>Aspergillus</u> <u>niger</u>	10		11
<u>Aspergillus</u> spp.	10	6	4
<u>Paecilomyces</u> <u>varioti</u>	9	5	4
<u>Penicillium</u> <u>herquei</u>	9		9
<u>Paecilomyces</u> spp.	9	6	3
<u>Penicillium</u> <u>ochrochloron</u>	8	1	7
<u>Cephalosporium</u> spp.	8	3	5
Dematiaceae	7		7
<u>Gonytrichum</u> <u>macrocladium</u>	7	2	5
<u>Aspergillus</u> <u>ustus</u>	6		6
<u>Penicillium</u> <u>canescens</u>	6	4	2
<u>Penicillium</u> <u>frequentens</u>	6	1	5
<u>Trichosporon</u> sp.	5		5
<u>Mucor</u> <u>angulisporus</u>	4	2	2
<u>Sporotrichum</u> sp.	4		4
<u>Acremonium</u> sp.	3	3	
<u>Alternaria</u> <u>tenuis</u>	3	1	2
<u>Aspergillus</u> <u>carneus</u>	3	2	1
<u>Gliocladium</u> <u>roseum</u>	3	2	1
<u>Penicillium</u> <u>decumbens</u>	3	2	1
<u>Penicillium</u> <u>spinulosum</u>	3		3
<u>Aspergillus</u> <u>ochraceus</u>	2	1	1
<u>Aspergillus</u> <u>sydowii</u>	2		2
<u>Aspergillus</u> <u>versicolor</u>	2		2
<u>Aspergillus</u> <u>wentii</u>	2		2
<u>Chloridium</u> <u>chlamydosporum</u>	2		2
<u>Gliocladium</u> sp.	2	1	1
<u>Paecilomyces</u> <u>marquandii</u>	2	1	1
<u>Penicillium</u> <u>nigricans</u>	3	1	2
<u>Penicillium</u> <u>oxalicum</u>	2		2
<u>Penicillium</u> <u>sclerotiorum</u>	2		2
<u>Rhizopus</u> sp.	2		2

Table 75. p. 2

<u>Ascomycete</u>	1	1	
<u>Aspergillus repens</u>	1	1	
<u>Aspergillus tamarii</u>	1		1
<u>Chrysosporium pannorum</u>	1		1
<u>Cladosporium herbarum</u>	1	1	
<u>Curvularia sp.</u>	1	1	
<u>Isaria farinacea</u>	1		1
<u>Memnoniella echinata</u>	1		1
<u>Mortierella isabellina</u>	1	1	
<u>Paecilomyces fumoso-roseus</u>	1	1	
<u>Penicillium charlesii</u>	1		1
<u>Penicillium levitum</u>	1		1
<u>Penicillium martensii</u>	1		1
<u>Penicillium meleagrinum</u>	1	1	
<u>Penicillium restrictum</u>	1		1
<u>Penicillium stipitatum</u>	1	1	
<u>Pythium sp.</u>	1	1	
<u>Scopulariopsis brevicaulis</u>	1		1
<u>Stemphylium sp.</u>	1		1
White yeasts.	1		1

from a pulping mill not only increases the number of species but also increases the rate of growth of the existing fungal biota of the soil markedly. This increase in numbers of colonies appears to be correlated with a decrease in the amount of BOD in the waste, which is inhibitory to the normal Actinomycete population in the soil but has little effect on the numbers of bacteria present.

Of the segments of the fungal populations studied, the greatest increase was in the numbers of species of yeasts. The numbers of species of filamentous fungi are affected to a lesser extent; however, there is a marked change in the distributions of the mold species in the soil and of the species able to tolerate the new growth medium and make use of it.

Knowing the source of the cells on which these counts are based would be interesting. From isolations made to date in a variety of habitats from a number of areas, it appears that areas in which high moisture and organic matter levels are maintained have larger yeast populations than dry areas that retain their natural supplies of organic matter. Certain industrial wastes can aid in the development of larger populations of yeasts, especially wastes rich in sugar. The original inoculum may have come from the natural populations in the soil, or it may have been brought in the air spora continually settling out on the ground or on any surface. Also, the original inoculum may have been brought in through the addition of domestic wastes and fecal pollution, although in the wastes from Niagara the latter factor was probably minimal or perhaps nonexistent.

C. Effect of fungi on waste materials containing lignin residues*

A number of tests with microorganisms have been made in various laboratories in attempts to get organisms which can degrade lignins in pure culture. It has been assumed for some time that lignin is destroyed in nature. Fungi are usually thought of as agents of this process. A preceding section has shown that under natural conditions lignin is reduced by fungi. Recently it has been reported by Burges (1958) that it takes three years for fungal activity to reduce lignin-containing materials and lignin itself to the almost completely insoluble and indestructible humic acids with which all are familiar.

In the laboratory, the commonest white-rot fungus has been shown by Day, Gottlieb and Pelczar (1953) to be unable to attack lignin or lignin-waste products. Those bacteria which have been commonly isolated from lignin-bearing substances such as composts, wood wastes, etc., have been shown by several groups of workers to be unable to attack lignin in any form in which it was subjected to attack: Adams and Ledingham (1942); Reynaud (1956); Tabak, Chambers and Kabler (1959). In all of these experiments it has become so abundantly clear that the lignin has not been destroyed but only adsorbed onto the cells in the cultures, that the experimenters have become discouraged with this method of attack and have gone on to more rewarding projects.

At intervals throughout several years prior to 1961, the Fungus Studies Laboratory at the Sanitary Engineering Center, has been interested

*Reproduced with permission from Proceedings of the 15th Industrial Waste Conference, Purdue University Engineering Bulletin 45:35-48, 1960 (1961); and TAPPI 46:573-578, 1963. Portions of each article were adapted for this presentation.

in trying to grow fungi on lignin-containing materials as sole carbon sources in the hope that a mold could be found which would degrade such complexes as spent sulphite liquor, waste Kraft liquor, Indulin and Meadol. Cultures were obtained from natural substrates of several fungi which, in nature, produce white-rots of decaying wood. Such fungi utilize the lignin in the natural wood leaving part of the cellulose and little else.

Cultures were grown for varying periods of time in liquid media in still and shaken culture on various concentrations of Indulin A, supplied by the West Virginia Pulp and Paper Company, on Meadol, supplied by the Mead Corporation, and on waste Kraft liquor supplied by the Champion Paper and Fiber Company. Growth of fungus mycelium was obvious in all cases, the quantity being small but dependent in part on the concentration of lignin-containing materials used and on the amount of nitrogen supplied. However, when the supernatant of each test run was checked by use of a Beckman DU spectrophotometer, results were erratic - those obtained by visual light were different from those obtained using ultraviolet light. Microscopic examination of the mycelium produced in these cultures indicated that in many cases a layer of material had been laid down on the surface of the hyphae.

In making primary isolations from the soil samples described above, it was noted that the phenolic dye in rose bengal agar was cleared by most colonies of Aspergillus niger which had appeared. This fungus had formed the bulk of the populations of molds isolated from the 10 ft level sample taken in 1958. A test was run in which 12 strains of black Aspergilli were grown in two levels of waste Kraft liquor, one with ammonium nitrate and one with neopeptone as nitrogen sources. Three of the cultures

gave what appeared to be good growth in this combination of nutrients. However, on testing the supernatant, and an aqueous extract of finely ground mycelium, it was found that no real loss of lignin or lignin-like materials had occurred. Lignin and lignin-like materials in the nutrient solution had only been adsorbed onto the mycelium.

Summary and Conclusions

It has been shown that the spraying of spent sulphite liquor onto a natural bed of coarse sand has been an adequate method of handling excesses of polluting materials during low water periods on a nearby river. The spray disposal of such wastes had added to the soil of a 40-acre field a quantity of nutrients which have favorable effects on the natural fungal populations, an apparently neutral effect on the natural bacterial populations and an unfavorable effect on the Actinomycetes populations. The effect on higher plants is unknown. Lignins or lignin-like materials tend to build up at various levels in the soil as leaching carries them downward. The only effects of such build-ups are slight temporary inhibition to certain members of the population of microorganisms present in the soil. These organisms appear to be capable of removing many of the simpler compounds which may be present in solution or in a colloidal suspension in the spent sulphite liquor. This statement is based on the fact that the populations build up with the increased spraying and the liquid which seeps out of the sand bed into the river appears to be free of important quantities of BOD-causing materials.

Fungi in Paper Mill Slime Accumulations and Wastewaters

Of greater immediate interest to the operator of a paper pulp mill are those fungi which hinder the process of paper making. It has been known for a number of years, according to the bibliography of Malin and Nannfeldt (1934), that fungi are capable of producing slimes in paper pulping processes which can be expensive nuisances. However, Malin and Nannfeldt (1934) themselves, were more interested in those fungi which cause "blueing" in the pulp itself. These include some of the same species which cause blueing in debarked logs, rough sawn boards, and finished lumber under the proper climatic conditions. These authors reviewed this situation carefully and listed and described a number of fungi which they found to be involved in the production of stains in pulp. These stains must be removed by discarding a batch of pulp or by expensive bleaching processes.

In the last 10 years interest has quickened in the problem of the causal agents of paper mill slimes. Brewer (1958, 1959a, b, 1960), in Nova Scotia, isolated 11 or more species of molds from slimes which he found. Those which seemed to be responsible for the most difficulty or were consistently present in his samples were studied physiologically. He found that the differences between isolates of one species from the same slime were as great as between isolates from different mills. In a second physiological study it was found that there were growth stimulating factors in white water. The substances were not identified but their effects were lessened after passage of the white water through resins or activated carbon.

Wang (1961) surveyed the populations of pulp and paper samples from 4 paper mills in New York State. She listed 42 species of filamentous fungi and discussed their distribution in pulp, paper and slime accumulations. Later, Wang (1965) published a bulletin in which 86 species of filamentous fungi and yeasts, and 7 species of Actinomycetes are described and illustrated. This bulletin was based on her work with the 4 paper mills mentioned above. It is the first comprehensive treatment of organisms from this habitat and should aid future students of the problem of slime control in their studies.

In the Stockholm area some paper mills are served by sewage treatment plants which also treat other municipal wastes. Rydholm (1962) was interested in the effect of the paper mill wastes on the populations of these plants and on the efficiency of the plants. Only two species of fungi are listed. Plant efficiencies were good. When paper mill wastes were added to other wastes levels of nitrogen and phosphorus had to be increased if good operation practices were to be continued.

X. Pollution Fungi in Human and Plant Disease

A. Isolation of potentially pathogenic fungi from polluted water and sewage *

In recent years, increasing evidence has accumulated that fungi pathogenic for man can be isolated from the environment. Ajello (1952, 1954), Cooke (1952), and Grayston and Furcolow (1953) have reported the finding of fungi which are considered etiologic agents of some of the deep-seated and the superficial mycoses of man, in habitats such as natural and cultivated soils; wild animal burrows; soils under nesting places of birds and fowl; undisturbed portions of buildings, such as attics, basements, and belfries where birds or animals nest, or roots and hollow trees.

A habitat type recently tapped for the presence of fungi, and which also is relatively rich in nitrogenous compounds, is sewage and polluted water. Here certain fungi occur, partly as members of natural communities, partly introduced by "run-off" or through excrements which form a large part of the habitat.

The presence of pathogenic fungi in any habitat does not necessarily imply the widespread occurrence of the diseases caused by them, nor does it imply the presence of a case of illness attributable to the disease in the immediate vicinity. In fact, records of diseases caused by the fungi to be discussed below are rare in the southwestern Ohio area where samples were obtained.

In addition to Allescheria boydii, which has been discussed by Ajello (1952), Aspergillus fumigatus and Geotrichum candidum have been

* Reproduced with permission from Public Health Reports 70:689-694, 1955.

isolated repeatedly. A. fumigatus is the etiological agent of one type of aspergillosis, a disease which has been described and illustrated in the textbook by Conant and his colleagues (1954). Reports in the literature dealing with soil molds, fungus contaminants, and cellulose deterioration indicate that this species is a very common fungus occurring throughout the world (Thom & Raper, 1945; Siu, 1951). It appears to be more common in warmer and more humid areas. Thus, it is not surprising that it would be found as a common mold associated with polluted streams and sewage disposal plants, where temperatures are usually higher than those of other parts of the environment, especially in colder months.

The pulmonary disease geotrichosis has been described and illustrated by Conant et al. (1954). G. candidum, also known as Oidium lactis or Oospora lactis, is the etiologic agent of this disease (Conant et al., 1954; Bobrov, 1951; Funder, 1953; Simons, 1954). It is also isolated frequently from sputum and other excretions from patients with other diseases, especially of the respiratory tract. The organism is one of the commonest fungi associated with the activities of man. It is the principal contaminant of dairies, is commonly associated with molds of milk and cottage cheese and other milk products, and may be one of the organisms involved in the manufacture of certain types of soft cheeses. It is a common laboratory contaminant and is frequently found in soils.

The fungi to be discussed below were found during routine examinations of polluted streams and sewage treatment systems described in the early chapters of this report.

Results and Discussion

Among the resulting fungus isolates were several cultures of a grey mold, some of which bore bodies which were at first thought to be pycnidia. Further study of young cultures on Leonian's agar showed that these were perithecia containing evanescent asci. These cultures were sent to Roy F. Cain, University of Toronto, who identified the fungus as A. boydii, a fungus which causes a type of mycetoma referred to as Madura foot. Isolates of this grey mold which did not produce perithecia were identified as Monosporium apiospermum which Emmons (1944) demonstrated is the imperfect state of A. boydii.

More recently, Ajello, in laboratory reports dated October 21, 1954, confirmed pathogenicity through mouse passage tests.

Allescheria boydii has been recovered 37 times and has appeared in 22 colony types on rose bengal agar. The 22 colony types show some polymorphism in the vegetative state, which can be grouped under variations of the following pattern: usually floccose or cottony; rather compact but sometimes rather loose; usually white and with white mycelium reaching above the main colony and spreading around its periphery, with cream to pale or olivaceous-green shades in the center of the colony and under the white hyphae. When the Allescheria phase was isolated directly, these colonies had perithecia in them. Perithecia were not recognized as such partly because the asci break down soon after spore formation and the whole mass appears as a pycnidium with abundant spores. Within the several types of media on which the fungus appeared, there was no significant difference in colony appearance,

and there was no significant difference between colonies obtained in winter or in summer. In stock cultures, in which the mycelium is grey instead of white, there seems to be a better production of perithecia on dextrose-phytone agar than is indicated in the literature for this species.

The occurrence of this organism in southwestern Ohio isolations in 1952 and 1953 is presented in Tables 76-78. From the evidence presented, it appears that A. boydii is able to survive and possibly to develop in beds of polluted streams, and in sludges in sewage treatment systems. It also appears on trickling filters in warm months. The numbers of colonies cited indicate a theoretical maximum rather than an actual number since the spore or mycelial fragment (or spores or mycelial fragments) which developed colonies on the primary isolation plates could have been the only viable propagules present in the 100 ml sample obtained from the habitat sampled. The fungus appears to develop more readily in the spring and summer months, although it has been isolated at other times of the year. No isolations have been made to determine whether or not other reservoirs of this fungus occur in southwestern Ohio.

From this series of isolations it appears possible that A. boydii is a member of the natural population of organisms in sewage and in polluted and fairly clean parts of streams. It further seems probable that the fungus is a member of the natural population of the soil, not only from reports of isolations by Emons (1950) and by Ajello (1952), but also because the stream and sewage plants under study are fed in part

Table 76. Occurrence of potentially pathogenic fungi isolated from Lytle Creek, Clinton Co., Ohio, 1952-53.
(Number of colonies in thousands per gram dry weight of sample.)

Station and Fungus*	2-27	4-17	5-15	6-12	7-10	8-7	9-18	10-23	11-26	1-14	2-25	3-30
Station 1.0												
<i>Aspergillus fumigatus</i>	10	26	10	16	26	24	0	0	0	0	62	14
<i>Geotrichum candidum</i>	76	30	38	0	8	116	8	0	122	308	152	50
Station 2.8												
<i>Aspergillus fumigatus</i>	234	46	8	36	32	10	0	0	14	0	34	0
<i>Geotrichum candidum</i>	156	20	28	0	10	110	0	0	154	76	96	0
Station 4.2												
<i>Aspergillus fumigatus</i>	118	74	86	72	44	0	18	40	10	40	28	44
<i>Geotrichum candidum</i>	228	0	34	0	144	0	16	8	126	44	38	38
Station 5.2												
<i>Allescheria boydii</i>	0	0	0	0	0	0	30	0	0	0	0	0
<i>Aspergillus fumigatus</i>	108	108	38	46	8	0	8	0	10	60	54	30
<i>Geotrichum candidum</i>	74	20	10	10	0	0	0	36	42	58	42	182
Station 6.5												
<i>Aspergillus fumigatus</i>	118	24	138	0	0	0	10	0	0	0	44	20
<i>Geotrichum candidum</i>	46	416	56	0	10	0	0	0	490	20	230	98
Station 7.2												
<i>Aspergillus fumigatus</i>	100	20	40	30	8	32	0	0	0	68	20	14
<i>Geotrichum candidum</i>	540	50	38	308	20	132	300	318	154	96	342	280
Station 7.6												
<i>Allescheria boydii</i>	0	20	10	0	0	0	0	0	0	10	0	0
<i>Aspergillus fumigatus</i>	16	186	24	58	26	0	10	16	20	50	24	54
<i>Geotrichum candidum</i>	146	20	10	300	34	300	0	0	48	42	204	152
Station 8.7												
<i>Allescheria boydii</i>	0	20	0	0	0	0	0	0	0	0	0	0
<i>Aspergillus fumigatus</i>	172	24	124	30	10	24	8	40	20	30	20	20
<i>Geotrichum candidum</i>	316	30	18	20	0	100	0	50	348	24	14	20

* Stations numbered according to distance in miles from mouth of creek. Stations fall in following pollution zones: Clean water, 1/10, 2/8, 8.7; Zone of Recovery, 7.6, 4.2; septic zone, 5.2, 6.5, 7.2; point of outfall from Wilmington, Ohio, sewage treatment plant, 7.2. See also Cooke (11) and Gaurin & Tarswell (14).

Table 77. Occurrence of potentially pathogenic fungi isolated from various stations in the Dayton, Ohio, sewage treatment plant, 1952-1953.
(Number of colonies in thousands per gram dry weight of sample.)

Station and fungus	1952										1953	
	4-3	6-19	7-17	8-14	9-25	10-30	12-10	1-29	2-12	3-19	4-14	
Inhoff tank sludge												
<i>Allescheria boydii</i>	N.S.	130	0	1,430	0	150	0	680	170	0	0	0
<i>Aspergillus fumigatus</i>	N.S.	0	780	130	520	450	170	0	0	0	0	0
<i>Geotrichum candidum</i>	N.S.	1,170	10,920	8,060	2,600	17,700	11,560	10,710	17,000	3,740	2,550	0
Scrapings, surface stone, High rate filter												
<i>Allescheria boydii</i>	0	2,520	0	4,410	0	0	0	0	0	0	0	0
<i>Aspergillus fumigatus</i>	630	0	7,560	0	2,520	0	0	0	0	0	0	0
<i>Geotrichum candidum</i>	7,560	5,670	7,560	111,510	31,500	162,478	137,780	24,900	310,420	46,480	25,730	0
Scrapings, surface stone, Low-rate filter												
<i>Allescheria boydii</i>	0	0	0	630	0	0	0	0	0	0	0	0
<i>Aspergillus fumigatus</i>	1,260	0	4,410	0	0	0	0	0	0	0	0	0
<i>Geotrichum candidum</i>	5,670	8,190	5,670	18,270	31,500	96,250	28,220	946,200	37,350	3,320	7,470	0
Wet, freshly poured digested sludge												
<i>Allescheria boydii</i>	0	54	0	4,536	0	130	0	0	0	72	N.S.	0
<i>Aspergillus fumigatus</i>	0	0	108	270	108	62	216	0	0	72	N.S.	0
<i>Geotrichum candidum</i>	324	324	432	1,620	0	6,804	4,536	144	3,816	2,808	N.S.	0
Medium-dry sludge on drying beds												
<i>Allescheria boydii</i>	0	0	0	570	0	204	0	80	0	0	0	0
<i>Aspergillus fumigatus</i>	0	0	0	30	0	34	80	80	40	0	0	0
<i>Geotrichum candidum</i>	60	90	270	30	0	408	880	480	4,000	0	360	0

yds

Table 78. Occurrence of potentially pathogenic fungi isolated from the Yellow Springs, Ohio, sewage treatment plant (activated sludge type), 1952-1953
(Number of colonies in thousands per gram dry weight of sample.)

Station and fungus	5-29-52	Date and Number of Colonies 7-31-52	10-9-52	3-11-53
First aerator chamber				
<u>Allescheria bordii</u>	0	0	0	0
<u>Aspergillus fumigatus</u>	0	0	5,000	0
<u>Geotrichum candidum</u>	125,000	22,500	45,000	49,500
Last aerator chamber				
<u>Allescheria bordii</u>	0	0	0	0
<u>Aspergillus fumigatus</u>	0	0	2,100	5,400
<u>Geotrichum candidum</u>	21,000	8,400	33,600	72,900
Digester sludge				
<u>Allescheria bordii</u>	0	0	660	0
<u>Aspergillus fumigatus</u>	0	250	0	0
<u>Geotrichum candidum</u>	20,000	2,750	7,000	9,280
Air drying sludge				
<u>Allescheria bordii</u>	260	810	770	0
<u>Aspergillus fumigatus</u>	0	84	0	0
<u>Geotrichum candidum</u>	10,080	0	4,284	56

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by natural drainage waters which could carry spores and other inocula from the soil to the stream and the sewage treatment plants of the area.

The isolations of A. fumigatus as to source and number of colonies are shown in Tables 76-79. This fungus appears to be less abundant in the more polluted portions of Lytle Creek and in the summer months. At the Dayton sewage treatment plant, it is abundant on the trickling filters in the cooler months, and it occurs commonly in both wet and drying aludges and in the Imhoff and secondary digester tanks. The complete picture of its occurrence at Dayton and Yellow Springs is not given since only sample stations selected as representative of the treatment processes are cited in Tables 77 and 78. Its sporadic occurrence may be a result of sampling error.

Table 79 shows that this fungus is present not only at Lytle Creek and at the Dayton and Yellow Springs sewage treatment plants, but in several other more or less polluted areas sampled only once or sporadically. Several samples taken along a stream draining the effluent from the Schenley Laboratories plant into Tanner's Creek near Lawrenceburg, Indiana, yielded large numbers of this fungus. It was found in a sample of Ohio River water, as well as in pool sediment and bank soil in the relatively clean stream, Cowan Creek, in southern Clinton County, and in bank soil along a clean creek draining the south slope of Fort Ancient, Warren Co., Ohio. It was found in sewage plant effluent at Ithaca, New York, in September, 1952, as well as in a sample of water taken from Cayuga Lake.

G. candidum was found to be one of the commoner species associated

Table 79. Occurrence of potentially pathogenic fungi isolated from miscellaneous sources, 1952-1953. (Number of colonies in thousands per gram dry weight of sample).

Location	Date	Fungus	
		<u>Aspergillus fumigatus</u>	<u>Geotrichum candidum</u>
Schenley Laboratories, Lawrenceburg, Indiana			
Effluent	6-26-52	4,300	0
	7-31-52	0	17,000
Gray Material on bank washed by stream	6-26-52	1,200	400,000
	7-31-52	0	420,000
Dry soil on bank above stream	6-26-52	400	1.2
	7-31-52	0	16.8
Creek water about 100 ft. below effluent outlet	6-26-52	2,000	1,000
	7-31-52	0	30,000
Glendale, Ohio			
trickling filter			
Influent	7-31-52	0	200
	3-11-53	0	280
Scrapings from surface stone	7-31-52	0	7,000
	3-11-53	0	4,200
Ohio River Water, Public Landing, Cincinnati, Ohio	6-26-52	3	3

with polluted water and sewage (Tables 76-79). It is exceedingly abundant on the trickling filters at Dayton, although it also occurs in other parts of the treatment plant. It is very common in activated sludge samples from aerators at Yellow Springs. In the small stream carrying the effluent from Schenley Laboratories to Tanner's Creek, it forms a mat on the creek bank as much as 1 cm. deep. It is common on the trickling filter at Glendale, Ohio, and was found in all areas sampled in the vicinity of the sewage treatment plant at Ithaca, New York. It occurred in the Ohio River and in riffle sediments and bank soil in the creek along the south slope of the Fort Ancient, Ohio, ridge.

G. candidum is a polymorphic fungus with many variant strains, none of which has yet been induced to produce a sexual state. It is possible that, of the many strains which exist, a few may be pathogenic. The many strains of this fungus are morphologically and physiologically fairly closely related so that, although a large number of names has been suggested, only one clearly defined species appears to be involved. In citing this fungus, most people have preferred to use the term "Geotrichum sp.". In a study with 16 strains, Bobrov (1951) was able to find only the type of germination in the arthrospore as a basis for segregation of three major strain types. She found she could not base species on this character. The group of strains she studied included cultures isolated from sewage, decaying leaves, and sour milk, as well as strains from suspected cases of geotrichosis in man and from sputum specimens from patients with other disorders.

From the data presented in Tables 76-79, it appears that these fungi

are relatively widespread in southwestern Ohio. Although A. boydii is not as abundant and not found as often as the other two fungi, this may be attributed to observational interpretation as well as to lack of inoculum because the colony types of A. fumigatus and G. candidum are more easily identified at sight.

Until recently, students of soil molds have been primarily concerned with potential or actual plant pathogens, or with saprobic organisms which break down organic matter. As improved techniques for the study of soil fungi become available and as more species are recognized, the occurrence in nature of fungi potentially pathogenic for man will be increasingly evident.

B. Potential Plant Pathogenic Fungi in Sewage and Polluted Water *

The literature of sewage and waste disposal contains many references to the use of sewage as a source of supplemental irrigation water. References are increasing as studies are made in drier parts of the country where sewage and other wastes are of increasing interest in reclamation of water.

Skulte (1956) states: "Skillfully designed and properly managed sewage farming has been operating for decades in Europe, and for several years in some localities in the southwestern areas of the United States, without danger to personnel and without creating nuisance conditions. As a new science, irrigation with sewage effluent is still in the experimental stage and many problems need to be solved in the fields of bacteriology, biology, hygiene, soil structure, and the physiology of plants."

* Reproduced with permission from Plant Disease Reporter 40:681-687, 1956. Some of this material was also used in Public Health Reports 72:651-654, 1957.

State and federal agencies have been interested for many years in the use of sewage as an irrigation supplement. Most communities in the United States could use supplemental irrigation (Hunt, 1954) during annual periods of drought. Methods of reclaiming sewage for reuse in irrigation have been described by Hutchins (1939), and are being studied by the State of California (Bush and Mulford, 1954; Merz, 1955). "Water", the Yearbook of Agriculture for 1955, shows in several articles both by Department of Agriculture personnel and by Public Health Service personnel an undercurrent of interest in this problem. On the research level, certain recent studies indicate some of the problems involved. The effect of spreading rather than furrowing in irrigation technique has been investigated by Bush and Mulford (1954) and by Greenburg and Thomas (1954). A study by Henry et al. (1954) was designed to indicate the influence of sewage waters on mineral content, organic content, soil structure, and other aspects of soil quality. In such studies it has been found that the most important mineral problem is that of boron which sewage seems to add to soils.

In a popular article by Stone (1955) it was pointed out that sewage could be used for irrigation in a number of places. In addition to irrigating crop plants, he thought, it could be used for the irrigation of lawns or oramental plantings. Illustrations of both types of use are given in his article.

Garrett (1956) indicates that until a crop is grown in a soil free from disease organisms one cannot appreciate the resulting gains in crop values or the typical healthy nature of the plants that develop in such

conditions. We may assume that this statement can also supply a contrast to soils into which disease organisms, however unapparent they may be, are dumped on a periodic and perhaps daily schedule.

Menzies (1954) has pointed out that certain plant pathogens have become more and more a problem to the plant pathologist as the practice of irrigation increases in the Columbia Basin. Those pathogens mentioned are airborne leaf and fruit diseases rather than water-borne soil fungi and either have been introduced with the crop or have migrated in much the same manner as other rusts, mildews, and host-restricted pathogens. His forecast that other diseases could become important in the region is of interest.

Two of the fungi isolated occasionally from sewage and polluted water are Fusarium oxysporum and F. solani. Strains of both have been recovered at one time or another from domestic sewage used as "seed" in experiments at the Robert A. Taft Sanitary Engineering Center. To determine the degrading ability of sewage organisms for various types of industrial wastes, small amounts of such sewage are seeded into dilution water containing known quantities of the wastes or materials to be studied. In one such experiment, hydrocarbons in the form of motor oil were subjected to domestic sewage. During the period of observation, two groups of organisms developed in large quantities, a protozoan and a strain of F. oxysporum. In a later experiment this strain of F. oxysporum was added to a suspension of motor oil in dilution water. After three weeks (Ludzack & Kinkead, 1955) the fungus had produced almost as much breakdown of the oil as had the domestic sewage which was tested at the same time in similar conditions.

Similar sewage seed had been used to develop a slime on an experimental trickling filter in which hydrocarbons of airplane cleaning materials were treated. Upon plating the slimes it was found that a strain of F. solani was the predominant fungus present.

Prior to 1952, the presence of fungi in sewage and polluted water was considered by workers in the field of sanitary science to be an established fact but not worthy of special investigation. It was obvious that under certain conditions of pollution a stream or a trickling filter could develop growths thought to be fungi, some of which were identified to species in occasional studies. No reference to the isolation or identification of plant pathogens as such from raw sewage or partially treated waters has been reviewed.

The routine study of a small polluted stream, Lytle Creek, Clinton Co., Ohio, of a large secondary-type sewage treatment plant at Dayton, Montgomery Co., Ohio, at monthly intervals, and of other habitats at less frequent intervals, yielded semiquantitative and qualitative data on the presence of soil fungi in these habitats. Various media were tested and techniques used which are common to soil microbiology (see Chapter II). From such studies it appears that certain strains of species of soil fungi have become acclimated to living in aqueous habitats. Spore production is atypical when present, and certain tested species are able to use dissolved oxygen in competition with other sewage organisms for reduction of organic materials (see B.O.D. study Chapter XII-C). Most of the species tested are able to produce their own vitamin supply, and all are able to utilize simple sugars and organic as well as ammonia nitrogen.

Garrett considers seedling blight and vascular wilt fungi as "ecologically obligate parasites"; that is, while they are able to grow alone or with other fungi on culture plates, in the soil they appear unable to compete with strictly saprobic fungi for the dead organic matter that occurs there. On the other hand, there is evidence that in sewage and related habitats some strains can reproduce, as indicated in the observations on degradation of hydrocarbons.

In addition to the strains of the two species of Fusarium mentioned, other fungi have been found that are capable of attacking plants in the field, or plant products in the field, in harvesting, in storage, in transit, and on the market. Species in Table 80 have been isolated from polluted waters and sewages. Some of the species in this list may be of little importance, but all have been cited in one reference or another as causes of diseases in plants, associated with seed in storage, or causes of various types of rots or decays in storage, transit, and market. Most of the species in this list are mentioned prominently in "Plant Diseases", the 1953 Yearbook of Agriculture.

The fungi chosen for presentation in Tables 81 and 82 are among the commoner species that have been isolated from sewage and polluted water and have been listed in the literature of plant pathology as causing damage to growing plants or their products in the field, storage, transit, or market. Alternaria tenuis is listed in the broadest sense as including any isolate that has morphological characteristics assignable to the species. Thus, several species of perfect fungi may be included in this category. The three species of Aspergillus cited are commonly associated with various types of storage, transit, and market decays,

Table 80. Fungi of interest of the plant pathologist isolated from polluted water and sewage.

<u>Alternaria tenuis</u>	<u>Fusarium solani</u>
<u>Aspergillus flavus</u>	<u>Gliocladium catenulatum</u>
<u>Aspergillus fumigatus</u>	<u>Gliocladium roseum</u>
<u>Aspergillus niger</u>	<u>Mucor - Several species</u>
<u>Aspergillus terreus</u>	<u>Penicillium brevi-compactum</u>
<u>Botrytis cinerea</u>	<u>Penicillium chrysogenum</u>
<u>Cephalosporium spp.</u>	<u>Penicillium cyclopium</u>
<u>Chaetomium funiculosum</u>	<u>Penicillium digitatum</u>
<u>Chaetomium globosum</u>	<u>Penicillium expansum</u>
<u>Cladosporium cladosporioides</u>	<u>Penicillium italicum</u>
<u>Coniothyrium fuckelii</u>	<u>Penicillium marzensii</u>
(and other Sphaeropsidaceous fungi)	<u>Penicillium oxalicum</u>
<u>Curvularia lunata</u>	<u>Penicillium rugulosum</u>
<u>Fusarium aqueductuum (epispheeria)</u>	<u>Rhizopus nigricans and other species</u>
<u>Fusarium moniliforme</u>	<u>Scopulariopsis brevicaulis</u>
<u>Fusarium oxysporum</u>	<u>Stemphylium consortiale</u>
<u>Fusarium roseum</u>	<u>Trichoderma viride</u>

Table 81. Occurrence of potentially plant pathogenic fungi isolated from Lytle Creek, Clinton Co., Ohio
(Number of colonies in thousands per gram dry weight of four combined samples
at each station in stream - and 3 pairs of stations combined.)

Station* and fungus	1952										1953		
	3-3	4-17	5-15	6-12	7-10	8-7	9-18	10-23	11-26	1-24	2-25	3-30	
Station 8.7													
<i>Alternaria tenuis</i>	8	0	0	0	0	0	0	0	0	0	0	0	
<i>Aspergillus fumigatus</i>	172	20	124	30	10	24	8	40	20	30	20	20	
<i>Aspergillus niger</i>	0	8	8	10	0	0	0	0	0	14	0	0	
<i>Fusarium oxysporum</i>	0	0	0	18	0	0	0	0	0	0	0	0	
<i>Fusarium solani</i>	0	0	10	0	0	0	0	0	0	54	0	0	
<i>Penicillium oxalicum</i>	0	0	0	0	0	0	0	0	34	0	0	0	
<i>Sphaeropsidales</i>	8	38	108	0	0	0	100	140	0	342	0	0	
Station 7.6													
<i>Alternaria tenuis</i>	0	0	0	0	0	0	0	24	0	40	62	80	
<i>Aspergillus fumigatus</i>	16	186	24	58	26	0	10	0	20	50	34	54	
<i>Aspergillus niger</i>	8	0	16	10	0	0	0	72	520	444	50	0	
<i>Fusarium oxysporum</i>	18	0	0	0	0	0	0	0	0	0	0	0	
<i>Fusarium solani</i>	0	0	0	0	0	0	0	0	0	24	0	0	
<i>Penicillium oxalicum</i>	0	0	0	0	0	0	0	16	70	0	0	0	
<i>Sphaeropsidales</i>	32	36	0	0	0	16	20	8	14	202	10	0	
Stations 7.2 + 6.5													
<i>Alternaria tenuis</i>	0	0	8	0	10	0	0	100	34	44	0	28	
<i>Aspergillus fumigatus</i>	218	44	178	30	0	32	10	3216	420	68	64	34	
<i>Aspergillus niger</i>	0	0	0	8	0	0	100	0	0	10	0	0	
<i>Fusarium oxysporum</i>	0	0	0	32	0	0	88	0	0	14	0	0	
<i>Fusarium solani</i>	0	0	48	0	8	0	0	0	0	0	0	0	
<i>Penicillium oxalicum</i>	0	0	108	0	0	0	0	42	1530	20	0	0	
<i>Sphaeropsidales</i>	16	336	26	0	0	100	8	24	30	474	114	0	
Stations 5.2 + 4.2													
<i>Alternaria tenuis</i>	0	0	0	0	0	0	0	0	10	30	70	74	
<i>Aspergillus fumigatus</i>	226	182	124	118	52	100	26	40	20	100	82	74	
<i>Aspergillus niger</i>	0	26	0	0	10	0	100	0	10	0	0	14	
<i>Fusarium oxysporum</i>	0	0	0	0	0	20	56	0	0	0	0	0	
<i>Fusarium solani</i>	0	0	0	0	0	0	0	0	0	0	0	0	
<i>Penicillium oxalicum</i>	0	0	0	0	0	8	104	100	60	54	0	0	
<i>Sphaeropsidales</i>	34	40	0	0	0	0	112	290	54	2868	164	10	

Table 81. p. 2

Stations 2.8 + 1.0	0	72	8	8	0	34	10	0	24	10	406	100
<u>Alternaria tenuis</u>	244	8	16	52	58	34	0	0	14	0	96	14
<u>Aspergillus fumigatus</u>	500	8	0	0	200	8	108	0	14	0	68	0
<u>Aspergillus niger</u>	6	0	0	36	0	10	0	0	0	150	0	0
<u>Penicillium oxysporum</u>	0	0	0	0	0	0	0	0	0	1374	0	0
<u>Penicillium solani</u>	0	0	0	0	30	0	0	0	0	10	24	68
<u>Penicillium psallidum</u>	0	8	10	10	56	216	16	108	150	10	0	0
<u>Sphaeropsisales</u>	0	0	146	0	0	18	36	268	52	100	118	64

* See Table 76.

Table 82. Occurrence of potentially plant pathogenic fungi isolated from the sewage treatment plant, Dayton, Ohio. (Number of colonies on thousands per gram dry weight of sample.)

Station and Fungus	1952										1953		
	4-3	6-16	7-17	8-14	9-25	10-30	12-10	1-29	2-12	3-19	4-14	5-19	6-19
Inhoff Tank Sludge													
<i>Alternaria tenuis</i>			0	0	0	0	0	0	0	100	0	100	100
<i>Aspergillus flavus</i>		150	450	0	225	172	0	100	100	100	200	100	100
<i>Aspergillus fumigatus</i>		600	150	0	450	258	200	200	100	200	100	0	0
<i>Aspergillus niger</i>		600	150	0	0	258	0	0	0	0	0	0	0
<i>Penicillium solani</i>		0	0	0	0	86	0	0	0	0	0	0	0
<i>Penicillium oxalicum</i>		0	0	0	375	0	0	0	200	100	0	0	0
<i>Sphaeropsis</i>													
Inhoff Tank Sludge													
<i>Alternaria tenuis</i>		0	0	0	0	0	0	0	0	170	0	170	170
<i>Aspergillus flavus</i>		0	260	0	0	0	0	0	340	0	0	0	0
<i>Aspergillus fumigatus</i>		0	350	0	260	300	170	0	0	0	0	0	0
<i>Aspergillus niger</i>		650	520	200	200	150	170	0	0	0	0	0	0
<i>Penicillium solani</i>		0	0	1170	0	1800	0	0	0	0	0	0	0
<i>Penicillium oxalicum</i>		0	0	520	650	0	0	170	0	0	0	0	0
Secondary Digester Sludge													
<i>Aspergillus flavus</i>		0	220	0	0	0	0	0	0	0	0	0	0
<i>Aspergillus fumigatus</i>		110	220	0	110	0	0	0	150	0	0	0	0
<i>Aspergillus niger</i>		110	350	110	0	130	0	0	0	0	0	0	0
<i>Penicillium solani</i>		0	330	0	0	0	0	0	0	0	0	0	0
<i>Sphaeropsis</i>		0	0	1100	4070	0	0	0	0	0	0	0	0
Scrappings, High Rate Trickling Filter Stone													
<i>Alternaria tenuis</i>	0	0	0	0	0	0	0	0	830	830	0	0	0
<i>Aspergillus flavus</i>	0	0	0	0	0	1540	0	0	0	830	0	0	0
<i>Aspergillus fumigatus</i>	630	0	2520	0	2520	0	0	0	0	0	0	1660	1660
<i>Aspergillus niger</i>	0	0	630	1260	1890	770	1660	830	0	0	0	830	830
<i>Penicillium oxalicum</i>	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Sphaeropsis</i>	630	0	0	1260	0	0	0	0	0	1660	830	0	0
Scrappings, Low Rate Trickling Filter Stone													
<i>Alternaria tenuis</i>	1260	0	0	0	0	0	1660	0	1660	830	0	2480	2480
<i>Aspergillus flavus</i>	0	0	0	630	0	0	0	0	1660	0	0	0	0
<i>Aspergillus fumigatus</i>	1260	0	1890	0	0	0	0	0	0	0	0	0	0
<i>Aspergillus niger</i>	0	0	0	630	630	770	830	830	0	0	0	830	830
<i>Sphaeropsis</i>	2500	0	0	4440	3150	1540	830	830	830	0	1660	1660	1660

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[illegible]

and some are commonly isolated from seeds. Several strains of Fusarium oxysporum and F. solani may be present in the region, but which of these is involved in the sewage isolations is not known. In order to determine this properly the cultivated and wild flora of the area should be screened for susceptibility of attack by these fungi. Penicillium oxalicum is reported to cause a seedling disease of corn, and it is also isolated on occasion from ears and seeds. Its occurrence in the area is of interest, since corn is one of the important crop plants there. The category "Sphaeropsidales" includes between 5 and 20 species, depending on the amount of variation permitted to occur within one species of imperfect fungi. Such genera as Coniothyrium, Phoma, Sphaeroneura and Ascochyta are present, and one of the commoner species has been identified as Coniothyrium fuckelii.

The fact that a number of species of these fungi are able to survive under conditions of extreme pollution, and even dilution of this pollution, may indicate a major adjustment in their life requirements. It may further indicate that they are probably aiding in the purification of the stream or the sewage. If they are aiding in stream purification by using as food some of the various types of pollutant materials, this may indicate that they are both increasing the size of their thallus and producing spores. The production of spores in situations like this, which are subject to occasional tapping for irrigation water, at least in some parts of the country, indicates that here is a potential source of inoculum for plant disease and pest species. In many cases this source need be present only for a limited period of time.

Nine of the 16 sampling points studied in the Dayton, Ohio, sewage treatment plant are listed in Table 82. These 9 were chosen because they are locations where the largest buildup of fungus populations occurs or because they can be of importance as sources of fungus contamination through use as fertilizer or soil conditioner. While not listed, various effluents can be of some, although lesser, importance as indicated by Becker and Shaw (1955).

During the sampling period the flow chart at Dayton was similar to that of many secondary-type sewage treatment plants using trickling filters. This was described above.

In certain communities, sewages in various stages of treatment are used as sources of irrigation supplements. The raw sewage used can contain fungal spores introduced from such sources as runoff from soils following precipitation or lawn and flower bed irrigation; washings from infected or mouldy plant parts, mildewed materials, etc.; or contaminated plumbing where growths can occur between intermittent growth periods. In other communities, effluents from primary settling tanks or Imhoff tanks are used; and in some cases, secondary treatment processes may be bypassed. In still other communities, effluents from complete or secondary plants may be employed. Depending on the type of treatment, that is, whether recirculation is practiced or whether the plant is overloaded, the effluent may be rich or poor in fungal disseminules. There may be seasonal activity within the treatment plant, or in the various places from which its populations are derived, which would give a seasonal pattern to the microorganisms in its effluent. The sludges used for

supplemental fertilizers or soil conditioners may have developed a population of organisms that could produce trouble in the field or at least add to the populations of microorganisms in the field awaiting favorable conditions for infecting crop plants.

The paradox of this situation is that under certain circumstances a fungus that could cause considerable damage in the field could be actively purifying the sewage in which it is living. The test in which it was found that a strain of Fusarium oxysporum could reduce motor oil in dilution water is a case in point. Should a treatment for such wastes be developed in which this or similar fungi were the microbial agents, and should the effluents containing spores or other disseminules be applied as irrigation supplements indiscriminantly, it is likely that an inoculum for disease of a crop plant could develop and considerable economic loss result.

It thus appears that in these days of increased supplemental irrigation by use of sewage or polluted water, the plant pathologist may be presented with additional problems of disease control. Unfortunately, to control one fungus chemically may inhibit the work of many beneficial organisms, so that control measures for irrigation waters should be applied to effluents rather than to influents of the sewage treatment plant. It is possible that adequate control of plant pathogens may also result in adequate control of human pathogens, so that a wider use could be made of sewage effluents than is being made today.

Supplement

At the time the two above sections were originally prepared, identifications on many cultures and isolations from many habitats were incomplete.

The lists of species given for the various habitat-types sampled will list additional species of potential plant pathogens.

One of the commonest fungi which has been found in sewage, sewage polluted waters, soils, and all similar habitats sampled is a fungus lately known, although incorrectly, as Phialophora jeanselmei (Langeron) Emmons, now known as Rhinoctadiella mansonii (Cast.) Schol-Schwarz. From practically every habitat sampled on most types of agar media and some of the liquid media, this fungus has been found in moderate to large numbers providing the plates are retained for at least 7 days. It appears as small black colonies which may even appear as pinheads or pinpoints. This species is not known to cause disease in man in temperate regions but in the tropics it is considered to be the etiologic agent of mycetoma, a disforming and unsightly type of skin disease.

XI. Indicator Organisms

A. Use and Value of Fungi as Biological Indicators of Pollution*

For the purposes of this discussion, our immediate concern is based on whether or not fungi can be used as indicators of pollution. It has been said (Suter & Moore, 1922) that the Saprolegniaceae can be used as indicators of pollution. It has been said (Suter & Moore, 1922; Butcher, 1932) that Leptomitius lacteus is an indicator of pollution. The filamentous bacterium, Sphaerotilus natans, in its several forms, and the stalked protozoa including Carchesium have been referred to as fungi which indicate pollution (Butcher, 1932). That little interest has been shown in analyzing the components of "sewage fungus" may be seen in Wilson's paper on the microbicta of sewage published in 1944. Here, "fungi indet." is simply one category among the many types of organisms

* Reproduced from Transactions of a Seminar on Biological Problems in Water Pollution, R. A. Taft Sanitary Engineering Center, Cincinnati, O. pp. 23-27, 1956 (1957).

listed according to genus. The number and types of fungi found in polluted water and sewage to date have only indicated that certain strains of a number of common soil fungi have become adapted to or are able to tolerate this different habitat.

From the beginning of life, various members of the populations of the world have been leaving their bodies or the remains of their metabolic processes on the land or in the water to continue the process of decay so that the organic materials of which they were composed may be used and reused. One type of intermediate organism in the waters of the world composes the group commonly called "sewage fungus". These organisms developed a specialized type of metabolism in which certain types of carbon and nitrogen compounds can be utilized but not others. As the numbers of man increased, and as his physical requirements increased, the waters of the world have increasingly carried the waste products resulting from this development, and organisms adapted to the special nutrients found therein have thrived and multiplied. In this way, we may suppose, such organisms as Sphaerotilus and Leptomit have developed, multiplied, and filled their special ecological niches. Leptomit has developed to the point that it uses fatty acids rather than sugars, and amino acids rather than ammonia (Cantino, 1955).

There are at least three sources of pollution by which food materials usable by fungi are placed in streams. The first may be considered natural pollution. Pieces of decaying vegetable or animal materials may inadvertently reach streams through runoff, passive falling into the water, or other means. We are not concerned with this type of pollution.

Many of the true water molds or aquatic fungi - Saprolegniales and related fungi - are found only under these conditions. They require something to which to attach themselves during their vegetative phases. Such fragments of materials may serve as food as well as anchoring places for these water molds. Other water molds, belonging to the Chytridiales, do not always live freely in the water but may be parasitic upon various species of algae and in various kinds of pollen which float on the water usually in the spring. When their sources of nutrition become exhausted, these fungi produce resting spores and cells (some times referred to as "gemmae").

The second source of pollution is fecal material and other materials such as ground garbage added to the stream in domestic sewage. Such materials are high in organic content and may furnish large amounts of foods to fungi that have become adapted in one way or another to life in the water or in the stream bed. Under restricted conditions, with certain carbohydrate, nitrogenous, and as yet undetermined activities, and under certain conditions of substrate and bottom materials resulting in special pH and mineral content conditions, certain Saprolegniaceae, Leptomit lacteus, Sphaerotilus natans, and various soil fungi such as Fusarium aquaeductum, Geotrichum candidum, Penicillium lilacinum, and others, may thrive. Under these conditions, such organisms appearing in large numbers will indicate the presence of polluting substances which make the waters of the stream unfit for human and many industrial uses.

The third source of pollution is various types of wastes from industry.

Different types of industrial pollutants can produce varying conditions of growth in the vicinity of the point of discharge of the pollutant. Such pollutants will become diluted downstream. It is possible that, at the immediate outfall, oxygen supplies may be too poor and certain substances may be too toxic to support growth, but as oxygen increases and the toxic substances become diluted by increased flow, precipitation, or other factors, the toxic effect will be weakened and growth of various fungi and fungus-like organisms may be encouraged or enhanced. When the pollutant is organic, Leptomitius lacteus and the filamentous bacterium Sphaerotilus natans may thrive under such conditions; and at least Sphaerotilus will present several growth habits, mistaken as species, genera, or varieties in the literature, as the concentrated waste becomes more dilute.

Both Leptomitius and Sphaerotilus have been cultured in the laboratory after the proper combination of nutrients has been determined. Such combinations indicate only that these organisms have special requirements for sugars and compounds containing nitrogen. In nature such substances are present largely under conditions of pollution by organic wastes from the home or factory; in the wilderness, such substances may be present following death and during decay of plants or animals. It is quite possible that these organisms may be common around watering holes and other places where animals have congregated, where their remains or droppings may have produced local natural pollution.

Another type of fungus may be thought to be more nearly indicative of pollution, especially of the domestic type. In the forest we find these coprophilous fungi associated with the dung of wild animals, rarely

of man. In many cases these fungi are restricted to specific types of dung. Macor rumenianus is found on frog droppings; Pilobolus species are usually found on horse droppings. Other types of these fungi are usually found on the dung of any animal that uses plants as food. The spores of these fungi are so produced and discharged that they become attached to or glued to leaves of plants used as food by grazing or browsing animals. After a spore has passed through the alimentary canal of an animal, it is able to germinate, use the dung as food, and fruit quickly after the deposit of the dung. Ephemeral mushrooms like the inky cap and the common mushroom are edible, but since man cooks the mushrooms he eats, their spores are killed before ingestion. Few if any coprophilous fungi are associated with human feces, especially where this material accumulates as night soil or is carried away in sewers to rivers or sewage treatment plants. While Sordaria humana occurs on other types of dung under other names, the specific epithet indicates that man also can ingest such fungus spores and that under certain conditions these fungi can be recovered from human wastes.

Three types of fungi which may be placed in this physiological class have been found in sewage polluted water and on trickling filters. A species of Pilobolus (Harvey, 1952) was found at one station on Lytle Creek by use of hemp seed as an isolating medium. It was found again on the Glendale, Ohio, trickling filter. Intensive biochemical nutrition research (Hesseltine et al, 1953) has shown that this fungus can be propagated in the laboratory without ingestion and alimentation by an animal, but the technique has not been tried at Cincinnati. Ascodesmia sphaerospora Obriet has been found on a trickling filter at

Dayton, Ohio. Other collections examined by Dr. Christ came from a variety of types of dung from North America, Brazil, and Germany. It requires no special culture techniques or media but will grow on any medium used in culturing the so-called sugar-fungi.

In 1953, Nesseltime, studying the trickling filters at Pearl River, New York, found the fungus Subbaromyces splandens which in pure culture grows only on lima beans in water agar. This species has since been found on trickling filters at Dayton, Ohio, Pullman, Washington, Moscow, Idaho, (Becker & Shaw, 1955) and Rockford, Illinois. It is possible that this fungus may grow in other habitats with similar environments, such as on stones of streams polluted with domestic wastes, and on other trickling filter beds.

An organism can become adapted to one set of environmental conditions or another. In some cases the adaptation may be complete, so that the organism cannot tolerate any other conditions, but more often various degrees of adaptation may be attained by an organism. Moser (1949), studying the adaptations of certain fungi, mosses and seed plants to areas in the tyrolean forests which had been burned by fires ranging from large forest fires to small camp fires, developed the following set of terms to describe the degree of adaptation of the organisms to burned areas: anthracobiont species were found only on burned soils and are not known to occur elsewhere; anthracophilous species are found more commonly on burned areas; anthracoxenous species occur more commonly on unburned areas but will tolerate burned areas; while anthracophobic species will not grow on burned areas. This set of suffixes

can be used to describe other habitat requirements of the same or different species. Since "copro-" has been used commonly in the combination "coprophilous" for a fungus growing on dung, it might be confusing to use it in the set of terms I want. The Greek word "lym" means "filth". Then lymbiont species will grow only on or in the presence of fecal materials; lymaphilous species will grow commonly on such material but will also grow on other materials; lymaxenous species will grow commonly on other materials but will tolerate fecal materials; while lymaphobous species will not grow on or in the presence of fecal material. In this sense a coprophilous species may be either a lymbiont or a lymaphile. A lymaxene could be an organism which on occasion will colonize the substrate material, while a lymaphobe will not grow on such materials. The emphasis here is on nutrient requirements or tolerances, rather than on habitat types or tolerances as is the emphasis in the Kolkwitz (1950) system.

To equate this set of terms with those of Kolkwitz (1950), and Kolkwitz and Marsson(1903), the following parallels are suggested. Polysaprobies would include both lymbionts and lymaphiles. For use in connection with fungi, polysaprobe would be confusing since it implies the ability to live saprobically on any substrate. Alpha (or strong) mesosaprobies would include lymaphiles primarily, but lymbionts and lymaxenes could be expected to be found. Beta (or weak) mesosaprobies would include lymaphiles, but lymaxenes could be found more frequently than in the preceding categories. Oligosaprobies would include the lymaphobes and probably some lymaxenes and lymaphiles.

Indicators of fecal pollution will thus be found in the first two categories, lyxmbionts and lyxmphiles, rather than restricted to the first category. Coprophilous species connote only those species of fungi or other organisms growing on dung at the time of observation, usually restricted to dung, so the term is not useful for our purposes. Of the lyxmphilous species one must be careful that the habitat is described in more than one way. For instance, the fungus Fusarium aquaeductum was first described from wooden water pipes which under certain conditions could be plugged with its massive growth. At present, wooden water pipes are used infrequently; pure water systems have been rarely checked for the fungus, and it is abundant on trickling filters throughout the country, as well as in polluted streams.

Since all fungi require preformed organic matter as sources of foods, and since many fungi have become adapted to the sewage treatment system, the sewer, or the polluted stream, it is entirely probable that there is no one fungus indicator of pollution or of organic enrichment of any kind. It is more probable that a community of fungal organisms, responding to the enrichment of the habitat by readily available nutrient supplies, forms a more reliable indicator of pollution.

B.Saprobic Valency

The language of decay is not a completely satisfactory one. However, in discussing aspects of decay related to pollution, certain words have been used to the extent that their meaning has been traditionalized in the thinking of those who deal with the populations of

polluted waters and sewage. According to Webster's Third International Dictionary, "saprobic" or "saprophytic" means "living in or being an environment rich in organic matter and relatively free from oxygen (e.g., organisms living in sewage)". If one thinks only of the fecal portion of sewage he could substitute "lyma-" for "sapro-" as the prefix in either word, or if one thinks of an organism living on or in dung he could substitute the prefix "copro-". For a long time the term "saprophyte", or its more recent form "saprobe", has been in use for those organisms such as fungi and non-chlorophyllous vascular plants, which live in and produce decay of wood and leaves on the forest floor. While many of the same fungi, as well as a few others, live in and produce decay in sewage and organically polluted waters, the habitat is much more restrictive and at the time the terms used in the preceding section were proposed it was hoped that the terms to be used could express this restriction. The terms suggested tried to define the role of the organism rather than the nature of the habitat, a feature, it was thought, of the Kolkwitz and Marsson saprobic system of habitat nomenclature. In presenting the list of fungi known from sewage and polluted water (Cooke, 1957) for each of the 147 species of fungi listed from 891 samples tested to that time, saprobic valency evaluations were included.

The original work on the saprobic system for describing the ecology of plants and animals in streams was first published in 1908. It was summarized briefly and brought up to date in 1950 by Kolkwitz. Four species of fungi are included among the alpha-mesosaprobies together with fair lists of bacteria and lengthy lists of algae for all saprobic classes in the latter work (Kolkwitz, 1950).

Sladetek (1963) in Prague, listed and illustrated 830 limnosaprobic plants and animals, giving saprobicity values for each species. Following a group of 36 species of bacteria illustrated on three plates, there are 15 plates of all types of algae. Plate 19 illustrates 12 species of fungi and 6 species of Bryophytes. One plate illustrates vascular plants and animals from flagellates and amoebae to insects and fishes are illustrated on 10 plates. Saprobicity values, appended to the legends for the plates, are given in terms of the Kolkwitz and Marsson (1908) system.

Fjordingstad (1960, 1964) thoroughly reviewed the literature referring to various systems for the classification of microorganisms and macroorganisms as well as their aquatic habitats with special reference to benthos communities. Rather than adopt "lyma-" for the organism and "sapro-" for the habitat in descriptive work, Fjordingstad chose to use "sapro" for both. In his final evaluation of the available terms based on their usefulness for the description of populations and habitats of rivers in Denmark, he chose the following groups of terms:

A. for organisms: "1. Saprobiontic organisms, occurring only in the most heavily polluted water. 2. Saprophilic organisms generally found in polluted waters, but also within other biotopes and consequently, within certain limits, indifferent. 3. Saproxenic organisms are generally found in other - non polluted - biotopes, but may thrive even in the presence of pollution. 4. Saprophobic organisms, who cannot live in polluted water".

B. Two zones for industrial pollution, the chemotoxic zone where chemicals toxic to all organisms occur, and the chemobiontic zone with

special types of populations dependent upon the types of organisms present and their response to the particular organic or inorganic chemical pollution found.

C. Communities can be described on the basis of dominant or sub-dominant species, and these species can be considered without necessarily referring to other species present.

D. The habitat can be described according to the Kolkwitz and Marsson system but with additional zones which seem to be needed at least for Danish streams: I. The coprozoic zone, comprising undiluted fecal water as well as domestic sewage before entering a treatment plant; II, III, and IV, the alpha-, beta-, and gamma- mesosaprobic zones; VIII, the oligosaprobic zone; and IX, the katharobic zone.

Fjordingstad, (1965) listed 182 species of bacteria, algae (all classes) and fungi (3 species) which he has studied in the waters of Denmark. For each species he gives a thorough review of the habitats from which they were reported in North America and Europe, and he concludes the discussion of each species with a statement concerning their saprobic valency.

XII Some Activities of Fungi

A. Carbon/Nitrogen Relationships of Fungus Culture Media *

Introduction

In order to learn something of the quantity and the quality of a population of mold fungi present in a soil, water, sewage, or other type of sample, the usual procedure is to plate the sample in a quantity of agar, incubate it, and after an appropriate period of time observe the number and kind of molds and yeasts which appear. For special members of a population many kinds of media are in general use. For the general preliminary study of the population of a habitat or a sample of material, certain general use media have been developed. Occasional modifications have been introduced but basic formulae are essentially as originally proposed.

As a result of a study on isolation technique (Cooke, 1954), certain culture media have been prepared in dehydrated form for sale to laboratories where fungi are isolated from various habitats. Samples of these preparations were compared with media prepared in our own laboratory and certain differences noted. An attempt has been made to determine why the variations which have been noted should occur. This has resulted in the following discussion in which an attempt is made to show something of the effects of the relationships between carbon and nitrogen in culture media. Certain other elements present in culture media are included in the discussion where thought to be pertinent to the type of work being considered.

Throughout the following discussion it is understood that in certain situations the absence of one or another inorganic major or minor nutrient is limiting. However, in all "natural" and all "semisynthetic" nutrients

* Reproduced with permission from Mycopathologia et Mycologia Applicata 34: 305-316, 1968.

such elements are usually present as contaminants of the chemicals used in preparing the media. In fact, in certain chemicals used in preparing "synthetic" media these elements may be present in sufficient quantity that they seemingly need not be added, but their addition is essential to ensure their presence, except in the most critical studies of mineral element nutrition where refinement processes must be used.

C/N Ratios in Natural Habitats

The substratum itself is the most important natural medium in which fungi grow. When green manures or straw are added to soil to increase its fertility level, microorganisms grow rapidly at first, then either become dormant or return to a comparatively sluggish growth pattern. According to Millar & Turk (1943), and Lyon & Buckman (1948) the carbon/nitrogen ratios occurring under such circumstances are listed in Table 83.

That C/N ratios vary among soils, both as to individual local samples and as to a broad latitudinal range of samples is indicated by figures given by Fehér (1933) who showed that at 22 stations between 46°30' in Hungary, and 69°30' in northern Finland, ratios increased northward ranging from as low as 4.76 in the south to as high as 66.7 in the north.

Whipple, Fair & Whipple (1927) list information relative to the amounts of dissolved organic C and N in Wisconsin lake waters. This is given in Table 84 as milligrams of C and N per cubic meter of water.

C/N Ratios in Laboratory Media

Types of Media

Henrici & Emmons in Skinner, Emmons & Tsuchiya (1947) referred to the three principal types of media used in the laboratory for the growth and preservation of fungi as non-reproducible and synthetic media; Lilly &

Table 83. C/N Ratios of Soils and Soil Additives

	C/N ratio
Miller & Turk	
Fresh plant materials commonly added to soils	
Mature straw	80:1
Leguminous green manure crops	12-20:1
Humus in mineral soil	Approx 10:1
Lyon & Buckman	
Legumes and farm manure	20-30:1
Straw residues	70:1
Bodies of microorganisms	4-9:1
Surface soils	8-(9-12)-18:1
Becoming constant at	9-10:1

Table 84. Dissolved organic C and organic N in Lake Water ($\mu\text{g}/\text{m}^3$)

	Mean C	Mean N	C/N ratio
Lake Mendota - 14 samples	5800	393	14.8:1
Other Lakes - 14 samples	6600	460	14.5:1

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Barnett (1952) use the terms natural, semi-synthetic and synthetic media. A non-reproducible or natural medium is made from natural plant or animal products and cannot be duplicated because the exact chemical composition is not known. Such media include potato plugs, carrot sticks, prune agar, and agars and broths made from other natural or altered natural materials. Serum and blood agars would be included here as well as infusions or decoctions of hay, manure, soil or other materials. Malt extract, wort and corn meal agars also fall in this category. In all these cases the worker is dealing with products of natural materials which vary from one batch to another no matter how closely the operator tries to duplicate the extraction process.

A reproducible or semi-synthetic medium is also based on materials made from natural products. However, there is more control over the manufacturing process and certain standardized additives are used which allow for more control over the composition of the final product. However, such media cannot be reproduced exactly for reasons which will become apparent. Examples of such media include potato dextrose agar, any medium which includes hydrolysates or enzymatic preparations of soy, meat or cheese as well as dextrose or other carbohydrate carbon sources, and other media of which the formula includes components produced under relatively constant conditions from natural materials.

A synthetic medium, such as the Czapek-Dox formula, can be reproduced exactly in the laboratory every time its use is required.

There are two basic types of semi-synthetic media which are in use today in the laboratory for general screening purposes as well as for stock cultures held in living condition. Many variations of these are in use both for general work and for the isolation and culture of specific

organisms or groups of organisms.

Of these two types, one was suggested by Sabouraud, one by Waksman. As will be indicated during this discussion, there is little essential difference between these types, and their separation is basically traditional, rather than factual.

According to Henrici & Emmons (l.c.) the original Sabouraud "proof" agar was composed of 4% crude maltose of Chanut and 1% granulated peptone of Chassaing. Although these writers suggested that the formula be changed to a ratio of 2% glucose/1% neopeptone, dehydrated Sabouraud media sold by the supply houses still utilize the ratio 4% carbon source (maltose or glucose) 1% nitrogen source (neopeptone, Difco or polypeptone, BBL). Henrici & Emmons suggest that the medium with a 2:1 ratio of carbon source to nitrogen source be termed Sabouraud or American Sabouraud.

Waksman (1922) presented 37 years ago his formula for a medium to be used for the isolation and study of soil fungi:

Glucose	10.0 g
Peptone	5.0 g
KH ₂ PO ₄	1.0 g
MgSO ₄ .7H ₂ O	0.5 g
Distilled water	1000.0 ml

This medium should be acidified to a pH of 3.6--3.8 by the addition of 12--15 ml normal H₂SO₄ or H₃PO₄ to reduce the numbers of bacteria. Such a medium has been used essentially without change by those students of soils in North America following the Waksman technique. It has been modified only recently by Martin (1950) who substituted for acidification the use of rose bengal and antibiotics to reduce bacterial contamination. This medium was used successfully by Martin with streptomycin as the antibiotic for soils studies, and by Cooke (1954) with aureomycin as the antibiotic for

sewage studies. It will be noted that this medium contains carbon source and nitrogen source materials in a ratio of 2:1. However, only half as much of these ingredients is used here as in American Sabouraud medium.

Following the publication of a report on the use of various media in isolating fungi from sewage and polluted water (Cooke, 1954), both Difco and BBL¹) produced a dehydrated Rose Bengal medium. While the Difco medium contained 1% glucose and 0.5% neopeptone, the BBL medium contained 1% glucose and 1% polypeptone. A special sample was prepared by the BBL laboratory in which 1% glucose and 0.5% polypeptone were used. In comparative tests all three media gave comparable quantitative results. However, qualitatively the medium with the 1:1 ratio of carbon-nitrogen source materials was highly inferior to the other media.

Less information is available concerning the carbon and nitrogen content of certain crudely prepared materials such as malt extract and potato extract than the peptones for which a certain amount of analytical data are available. According to Kirk & Othmer (1952), the approximate composition of malt extract is:

Starch	50	-55	%
Sugar	8	-10	%
Total carbon sources	58	-65	%
Total nitrogen	1.8	-2.3	%
30-40% of total nitrogen			
is soluble thus:			
Total soluble nitrogen	0.54	-0.92	%

On the basis of total available nitrogen the C/N ratio of malt extract would be 14:1 while on the basis of soluble nitrogen the ratio would be 28:1.

In Albritton (1956) one finds that in nutrition studies 19.1% of the potato is considered to be carbohydrate while 2% is protein. On the basis of dehydrated potato meal, 5.9% is crude protein while 70.6% is nitrogen-free

extract. Here the ratio is less precise since it must be based on source rather than on elemental constituents of the source. Thus in the first instance the ratio of carbon source to nitrogen source is 9.6:1, while in the second it is 12:1. Between batches of potatoes and methods of preparation there would be a considerable fluctuation of C/N ratios among different batches of media. If to the first type of preparation 2% glucose were added, the C/N ratio would increase to 14.6:1.

An additional type of semi-synthetic medium may be mentioned since data are available concerning its composition. In the testing of cellulose cloth for the activity of fungi in its degradation, Siu (1952) has presented data concerning the amount of cellulose decomposed in ratio to the amount of nitrogen assimilated.

In Table 85 data for five fungi are presented first as the ratio of carbon source used to nitrogen assimilated, and second as the ratio of carbon to nitrogen used. In calculating this value it is assumed that the cellulose is composed of 40% carbon.

Peptone-based media

BBL and Difco each manufacture 7 types of peptones. These are enzymatic digests or hydrolysates of meat, soy bean meal or cheese. Up to a point these materials have been analysed and amounts of nitrogen, amino acids, vitamins, and macro- and micro-elements reported. Analyses of the Difco products appear in that company's manual (1953), while those of the BBL products have been listed in a mimeographed report. In addition to these sources, more recent information has been obtained in correspondence. Some of these peptones have been used in media prepared for use as dehydrated complete media, others appear in standard recommended formulae.

Based on percentage of total nitrogen reported for the several

Table 85. Cellulose decomposed; Nitrogen assimilated

	Carbon source: N used	C:N ratio
<u>Monosporium</u> sp.	25:1	10 :1
<u>Coccospora</u> sp.	30:1	12 :1
<u>Botryosporium</u> sp.	48:1	19.2:1
<u>Stachybotrys</u> sp.	49:1	19.6:1
<u>Mycogone</u> sp.	54:1	21.6:1

peptones (writer's file), we can calculate the C:N ratio of the Waksman and Sabouraud type media. In all containing glucose and maltose as the carbon sources, the amount of carbon used is 40% of the weight of the carbon source. Table 86 presents the C:N ratios for dehydrated or laboratory prepared media in which the ratio of carbon source to nitrogen source material is 4:1, 2:1 or 1:1.

Following the nomenclature used for agars in describing these combinations of carbohydrates and peptones, all should be termed Sabouraud agars or media. In listing the dehydrated forms, various names have been used by the manufacturers, or in common use in the laboratory other names have been developed. The commoner names used for these media are listed in Table 87. If the nomenclature for media suggested by Lilly & Barnett (1952) is used, less confusion could result.

Synthetic or reproducible media

Three types of synthetic media may be mentioned here. These are chosen partly because they are used commonly in laboratories as the point of departure for the development of controlled test media and partly because at least one of them may be used at times for screening samples of material for mold populations. These media rely on asparagine, ammonia or nitrates as sources of nitrogen. They can be readily reproduced and many different chemicals can be added or their concentrations can be varied as the scope of the experiment changes. Carbon/nitrogen ratios of three such media are presented in Table 88.

C/N Ratios for Special Work

At a C:N ratio of 16:1 all the nitrogen present in a medium is used by fungi for cell synthesis. At a C:N ratio of 10:1 or 12:1 the medium is adequate for growth and for some synthesis of nitrogen-containing by-products.

Table 86. C:N ratios of culture media containing different C and N source materials

C source	N source	% C	% N	Ratios C source: N source	C:N
Glucose	Bacto-Peptone	40.00	16.16	2:1	5.0:1
Glucose	Gelysate BHL	40.00	16.0	1:1	2.5:1
Glucose	Soytone	40.00	9.5	2:1	5.0:1
Glucose	Phytone	40.00	9.5	1:1	2.5:1
Glucose or Maltose	Neopeptone	40.00	14.33	2:1	8.4:1
Glucose or Maltose	Polypeptone	40.00	12.15	1:1	4.2:1
				4:1	8.2:1
				1:1	4.1:1
				2:1	11.2:1
				1:1	5.6:1
				4:1	2.8:1
				2:1	13.2:1
				1:1	6.6:1
				1:1	3.3:1

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Table 87. Terminology of sugar-peptone media

Trade name	Carbon-nitrogen sources	C:N source ratio	C:N ratio
Sabouraud-Dextrose Agar (Difco)	Neopeptone, Glucose	4:1	11.2:1
Sabouraud-Dextrose Agar (BBL)	Polypeptone, Glucose	4:1	13.2:1
Sabouraud-Maltose Agar (Difco)	Neopeptone, Maltose	4:1	11.2:1
Sabouraud-Maltose Agar (BBL)	Polypeptone, Maltose	4:1	13.2:1
Mycophil Agar (BBL)	Polypeptone, Glucose	1:1	3.3:1
Mycophil Broth (BBL)	Polypeptone, Glucose	4:1	13.2:1
Mycosel Agar (BBL)	Polypeptone, Glucose	1:1	3.3:1
Sabouraud Liquid Medium (BBL)	Polypeptone, Glucose	2:1	6.6:1
Sabouraud Maltose Broth (BBL)	Polypeptone, Maltose	4:1	13.2:1
Rose Bengal Agar (BBL)	Polypeptone, Glucose	1:1	3.3:1
Sabouraud Maltose Broth (Difco)	Neopeptone, Maltose	4:1	11.2:1
Sabouraud Liquid Medium (Difco)	Neopeptone, Glucose	2:1	5.6:1
Mycological Agar (Difco)	Neopeptone, Glucose	1:1	2.8:1
Mycological Broth (Difco)	Neopeptone, Glucose	4:1	11.2:1
Rose Bengal Agar (Difco)	Neopeptone, Glucose	2:1	5.6:1
Rose Bengal Agar (2) (BBL)	Polypeptone, Glucose	2:1	6.6:1
Laboratory name			
Phytone-Dextrose Agar	Phytone, Glucose	2:1	8.2:1
Soytone-Dextrose Agar	Soytone, Glucose	2:1	8.4:1
Waksman's soil mold plate count agar (Difco)	Bactopeptone, Glucose	2:1	5.0:1
	Gelysate, Glucose	2:1	5.0:1

Table R8. Carbon/nitrogen ratios of three synthetic media

Medium	Carbon source/nitrogen source	Grams	C:N ratio
Glucose - L-asparagine agar	Glucose, L-asparagine	4.0:0.427	9 :1
Sucrose - Ammonium nitrate	Sucrose, ammonium nitrate	50.0:2.06	29.7:1
Czapek-Dox agar with glucose with sucrose	Glucose, sodium nitrate	30.0:3.0	21.2:1*
	Sucrose, sodium nitrate	30.0:3.0	42.3:1*

* Calculated on a percentage weight basis. If calculated on an atom-atom basis the values are 60:1 and 120:1 respectively.

Some reports in the literature indicate the C:N ratio required by fungi by biochemical syntheses. Foster (1949) reports that a low C:N ratio results in good growth in Rhizopus cultures but a high C:N ratio is required for rapid and effective conversion of sugar to organic acids. In other molds a high C:N ratio (8-15% carbohydrates) is needed for the accumulation of such acids. In those molds a low C:N ratio (0-0.5-2.0% carbohydrate) more nearly approximates natural conditions and all of the carbon is used in growth. Nitrogen must be present in sufficient quantity and in utilizable form. The presence of zinc is required for optimum growth and nitrogen utilization. Maximum sterol production in Aspergillus fischeri takes place at glucose: urea ratio of 21:1 or C:N ratio of 17.2:1.

Working with Penicillium chrysogenum, Stout & Koffler (1951) reported that in studies on endogenous respiration when 0.3% glucose was used in the medium, a ratio of 2-8 parts ammonium nitrate to 1 part glucose appeared to be optimum for glucose assimilation. At higher or lower concentrations of the nitrogen source a decrease in assimilation was noted. This is a C:N ratio of 1:1.75 to 1:7, requiring a larger amount of nitrogen and nitrogen source material than in other studies reported above.

C/N Ratios for Primary Isolation of Fungi

Several tests have been made in which different rose bengal agars have been used. This has been done to check commercially prepared materials against the formulation prepared in our own laboratory. In the first test, material prepared in our laboratory was compared with dehydrated material prepared by Difco of the same composition and by BBL in which neopeptone was replaced with 10 g polypeptone. The formula prepared at Cincinnati included the following ingredients:

Glucose	10.0 g
Neopeptone	5.0 g
KH_2PO_4	1.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
Rose Bengal	0.035 g
Agar	20.0 g
Water	1000.0 ml

In Table 89 results of plating 15 samples of different materials from the Dayton, Ohio, sewage treatment plant are summarized. The samples were collected Nov. 5, 1956, plated Nov. 6 and the results read Nov. 19.

As in earlier work (1954) different dilutions were used depending on the amount of solids in the sample. Numbers of colonies on 5 plates poured from each sample were totaled, the percent of the total for the three media was calculated and the average number of colonies per sample was obtained. In all cases aureomycin¹) (furnished through the courtesy of Lederle Laboratories) was used to suppress bacteria. The value for "range" indicates low and high values for each type of sample. For columns 1 and 3, low was obtained in final sewage, for column 2 in settled sewage. High values were all obtained in filtered sewage.

To determine if there were differences (Table 90) between the usefulness of neopeptone (14.33% nitrogen) and polypeptone (12.15% nitrogen), and if there were differences between these media in the presence or absence of added salts, a set of samples from the Dayton, Ohio, sewage treatment plant was plated using four media prepared at Cincinnati. Samples were collected May 21, 1957, plated May 22 and read May 31. This was done in replicates of four per sample. The antibiotic used was aureomycin. Twenty samples were obtained and dilutions were prepared as indicated earlier. Plates with low counts were all plated from heat dried sludge while high counts were obtained in three instances (1, 2, 4) from air dried sludge and in one instance (formula 3) from raw settled sludge. Formula 1 contained 10 g

Table 89. Total, percent of total, average numbers of colonies, and range obtained from samples of sewage treatment plant materials on three media

Category	Rose Bengal Agar Cincinnati	Rose Bengal Agar Difco	Rose Bengal Agar BBL
Total colonies	2374	2570	2178
Percent of total	33.3	36.1	30.6
Average number of colonies	158.5	171.5	145.2
Range	31-400	30-400	35-400
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Table 90. Total, percent of total, average number of colonies and range of colonies in samples from sewage treatment plant processes on four media

Category	Rose Bengal Agar with			
	Neopeptone No salts (1)	Polypeptone No salts (2)	Neopeptone Salts (3)	Polypeptone Salts (4)
Total colonies	1112	1051	1094	1067
Percent of total	25.7	24.3	25.3	24.7
Average number of colonies	55.6	52.6	54.7	53.4
Range	14-128	16-170	14-139	15-118

dextrose, 5 g neopeptone, 20 g agar in one liter of distilled water.

Formula 2 was the same but with polypeptone, formula 3 was like formula 1 but with 1 g KH_2PO_4 and 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and formula 4 was like formula 2 but with the same salts used in formula 3.

Discussion

Cochrane (1958) states: "In general, it has been too readily assumed that fungi need and tolerate high nutrient levels and high acidity; this is true of many fungi but by no means of all. For certain purposes, concentrated media are, of course, useful, but morphology, physiology, and reproduction are best studied in more dilute media than those often used."

Snyder & Hansen (1947) and Farrow (1954) have found that media in which plant parts are imbedded or which are based on nutrients with low concentrations supplemented by fragments of natural organic materials, are excellent media for isolation and cultivation of fungi. However, such media are natural and contain nonreproducible amounts of materials which are nutrients or growth promoters. The comments to be made here will refer only to semi-synthetic or synthetic media whose basic ingredients are known either so far as analyses have been made or because measured quantities have been brought together.

Studies on nutrients and proportions of nutrients present in synthetic and semisynthetic media have not progressed so far that a complete optimal medium can be cited for general primary isolation work. The synthetic media have not been used for general survey work to any great extent. Glucose-asparagine agar and sucrose-ammonium nitrate agar have been designed for special nutrition studies in the laboratory where it is necessary to shift various basic components of the formula to determine optimum concentrations. Czapek-Dox medium is designed to bring out certain characteristics

of such genera as Penicillium, Aspergillus and Paecilomyces in pure culture. This medium demonstrates the validity of the statement by Cochrane (1958): "Since endogenous respiration is affected by the composition of the growth medium, (one) approach would seem to be an attempt to find a medium in which the cells do not accumulate reserves in large amounts. Many of the media commonly used for fungi are so concentrated that accumulation of such reserves is almost forced upon the organism." With a C:N ratio of 42:1 in Czapek-Dox agar the organism is forced to build up reserves of carbon-containing materials. In this way certain characteristics become built in to a culture and these are used in part in identification. Such characteristics include pigmentation, water soluble exudates in the medium, dewlike exudates on the colony surface and certain growth characteristics.

In semisynthetic media C:N ratios are closer to reality, assuming that "reality" is approximately equal to the C:N ratio of soil materials or about 9:1 or 12:1. Some attempts to enrich medium to the extent of C:N ratios of 2.8:1 or 3.3:1 produce media which support good growth. However, this growth usually tells a person only something about the numbers of some molds. As the C:N ratio increases to 5.6 or 6.6:1 in media in which the ratio of carbon source to nitrogen source is 10:5, colonies become more discrete, more readily counted, more easily isolated for future independent study, and with familiarity, more readily identified at sight, at least to genus. This assumes, of course, that adequate dilutions have been made so that 40-60 colonies per plate can be observed. When ratios of carbon source to nitrogen source lie at 1:1 or 4:1 the factor of excess nutrients in the medium tends to befog the issue and counting or isolating procedures are difficult to carry out. It may be possible to obtain an adequate count of the plates made with such media, as in column 3, Table 89,

but the qualitative examination of the plate will be hindered, since colonies tend to be less discrete, coalesce, and merge. If it is assumed that $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ is added to the medium to give the fungi an adequate supply of magnesium and sulphur, and that KH_2PO_4 is added for enrichment of potassium and phosphorous, it should be noted that these elements have been identified in two of the peptones if not in the others, where they may be assumed to occur. By referring to Table 90 it will be seen that it is not necessary to add these compounds to the media. The results of simultaneous parallel plating on four media indicate that an adequate supply of these elements is present in the peptone materials used in preparing the media. With the use of rose bengal and antibiotics rather than a pH adjustment control of bacteria, many kinds of fungi have been found in the soil which were not previously reported. Of these, some appear to be of greater importance than some isolated at low pH values.

Summary and Conclusions

Four basic nutrient types are described and discussed in special reference to the ratios between carbon source materials and nitrogen source materials. These include habitat, natural nutrients, semisynthetic nutrients and synthetic nutrients. Special emphasis is placed on the use of such nutrients in agar media for the primary isolation of fungi from various types of habitat materials such as soils and sewage.

It is suggested that a basic C:N ratio for such nutrient agars lies at about 9 or 12 to 1, that increasing this ratio tends to over-enrich the culture thus encouraging the formation of intracellular or extracellular carbon-carrying compounds, and that decreasing the ratio tends to encourage the formation of nitrogen-carrying storage products. Both conditions tend

to decrease discreteness of colonies, tend to produce more diffuse, less easily counted growth, and tend to develop less readily identifiable colonies.

It is implied that if a C:N ratio more or less approximating that found in an agar containing plant parts, or limited nutrients plus plant parts, were used in a medium designed for primary isolation of fungi from natural materials, such a medium would have many advantages over currently popular media used for this purpose.

B. Nutritional Requirements of Nine Common Sewage Fungi*

An increasing amount of information is being compiled concerning the nutritional requirements of certain species or groups of fungal species. Such studies are usually confined to fungi of importance to a specified field of investigation such as commercial application, plant or human pathology, or comparative physiology.

Textbooks of fungus physiology (Hawker, 1950; Lilly and Barnett, 1951) describe the basic vitamin requirements and the types of sources of carbon and nitrogen which are metabolized by various fungi. Such fungi include those which have been used in extensive tests, and those about whose requirements and tolerances pertinent information is required for control for production of industrial products, or for use in assays of various chemical impurities, vitamin or antibiotic products. In addition, limited information concerning fungus nutrition is available in texts on industrial microbiology (Smith, 1954; Prescott and Dunn, (1949); Foster, 1949), and on mycology (Wolf & Wolf, 1947; Nickerson, 1947).

Within the last 25 years, research in this field has been given considerable impetus at the level of both general physiology and nutrition, in relation to the production specialist interested in increasing the output of specified products, and to the deterioration specialist interested in controlling unwanted growth of fungi on various types of agricultural and manufactured products.

In a recent review covering the vitamin requirements of fungi

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(Robbins and Kavanagh, 1942), emphasis has been placed on the information which was available up to 1942. The writers considered especially the ability of a fungus to synthesize vitamins essential for their metabolic processes. This ability of a fungus is either complete, incomplete, or absent. A vitamin deficiency may be complete, partial, or conditioned. Complete and partial deficiencies may be observed in certain species, and these may be single if they are for one vitamin, or multiple if they are for more than one. Only two of the fungi to be discussed were mentioned in a species-by-species review of many filamentous fungi representing all classes. Fusarium oxysporum was found (Robbins and Kavanagh, 1942) to grow well in its first passage in mineral sucrose medium to which KNO_3 or $Ca(NO_3)_2$ had been added. The same authors reported that Dematiium pullulans growth in mineral dextrose medium plus NH_4NO_3 was accelerated by the addition of camomile extract, and it also grew well in a mineral dextrose medium plus asparagine, with no effect noted after the addition of thiamine or an intermediate compound. Thus in neither case was the addition of vitamins required; both could be considered to have complete vitamin synthetic ability

Bobrov (1951) reported on the nutrition of Geotrichum candidum as one of the techniques used to find out whether specific variation occurred in the group of 15 strains which were being compared. These strains were isolated from a number of cases of human pulmonary disease as well as from various types of deteriorating natural materials. Auxanographic analyses were made with a basal medium containing 2% each

of glucose and agar for the nitrogen series and 0.5% $(\text{NH}_4)_2\text{SO}_4$ instead of glucose for the carbon series. In both series 0.1% KH_2PO_4 and 0.05% MgSO_4 were added. In cases where ammonium sulphate was unavailable to the fungus, an amino acid such as arginine, found suitable in the nitrogen study, was substituted in the same strength as ammonium sulphate.

Of the 15 strains tested, 13 gave good to excellent and dense growth in the presence of l-asparagine, ammonium sulphate, and urea as sole nitrogen sources, while growth was not obtained on either ammonium nitrate, calcium nitrate or potassium nitrate. In addition, zones of inhibition were formed in some cases around crystals of calcium nitrate when this chemical was added to the medium in crystalline form.

All of the 15 strains tested gave moderate to good growth on glucose and d-levulose as sole carbon sources. When d-xylose and d-mannose were employed, 13 of the 15 strains studied gave moderate to good growth. Only 3 of the 15 strains gave any growth on starch and this was poor to moderate depending on the strain, while on lactose 2 of the 15 strains gave only poor growth. No growth was obtained on sucrose.

According to all texts on fungal nutrition, most fungi require carbohydrates as their usual carbon source. Some species are able to metabolize pentoses, many prefer hexoses, some disaccharides, and some tolerate polysaccharides such as cellulose and starch. Occasional species can grow in the presence of carbon containing compounds when carbohydrate sources are lacking. These include proteins, keratin, some organic acids, and some kinds of fats. The second most important element required by fungi is nitrogen, partly because it is a fundamental constituent of protoplasm, and partly because it is one of the elements in the

chitin of the fungus cell wall.

Robbins (1937) has proposed four groupings of plant-like organisms based on their abilities to utilize various sources of nitrogen. Group I grows in the presence of molecular nitrogen which it uses, as well as other forms of nitrogen, and generally is not found among the fungi. Group IV grows only on organic nitrogen substrates, while Group III, in addition to organic nitrogen, can metabolize ammonium salts, and Group II develops on these two as well as on nitrates.

Most fungi require the same series of minerals in their nutrition as do all living things. Phosphorus, potassium, magnesium and sulphur are most important among these, and most synthetic media as well as many natural and semisynthetic media, include them in their formula. Chemicals used in preparation of synthetic and semisynthetic media contain as impurities substances which carry adequate amounts of these elements as well as the numerous trace elements required for "normal" growth and appearance.

The fungi chosen for the study reported below include the same series of species as those used in the following two studies. Because of the prevalence of these fungi in sewage and polluted waters, because of the ability of some to utilize wastes in water in competition with other organisms (Ludzack and Kinkead, 1956), and because some, if not all, form important segments of the slimes on trickling filters and the sludge in activated sludge, it was thought desirable to learn something of their nutritional requirements in pure culture.

Procedures

The procedures used in the series of studies on which the following results are based were essentially those outlined in the chapter on "Suggested laboratory exercises" in the text on fungus physiology by Lilly and Barnett (1951). Certain modifications in suggested techniques will be discussed here. Except for such stock solutions as microelements and vitamin solutions, all media were prepared the day before inoculations were made. Glassware was thoroughly washed with acid-chromate solution before nutrition incubation experiments were set up.

Stocks of inoculum for the experiments were prepared by inoculating a small amount of spores in 100 ml of phytone-dextrose broth and shaking for 3 days. The growth was filtered through a membrane filter, the nutrient solution discarded, and the mycelial mat resuspended in 100 ml sterile distilled water. The mycelium thus obtained was broken up in a Waring Blendor for 30 sec. at high speed, and stored in the refrigerator until needed. For Geotrichum candidum and Phialophora jeanselmei, the growths included single cells rather than mycelial masses so that blending was unnecessary. To prepare inoculum from these suspensions, 10 ml of the suspension was added to 100 ml distilled water. One ml of the resulting suspension was used as inoculum.

Rather than flasks, 100-ml square dilution bottles with plastic screw caps were employed for incubation of cultures. Twenty-five ml of the nutrient solution were inoculated with 1 ml of spore or cell suspension, the bottles coded, laid on their sides in stacks, and allowed to incubate at room conditions of temperature and light for at least two weeks. Harvests were made after 2, 5, 7, 9, 12 and 14 days by

collecting the mycelium in tared Gooch crucibles which were then oven-dried and weighed to obtain the dry weight of material produced by the fungus. Room temperatures varied between 20° and 25°C. during the period of the study.

Three principal types of nutritional requirements were studied. Vitamin requirements were considered first, and were followed by carbon and nitrogen assimilation experiments. For vitamin requirements, inocula which had been stored in sterile distilled water were added directly in the first two sets of studies. In these tests the glassware was triple washed with acid-chromate solution, once after use and twice before use. In the third test the glassware was washed with acid-chromate solution after use and rinsed with distilled water before use. The fungi were grown for three passages through media with the same composition (but without the vitamins) as that in which the fungi were to be exposed before being inoculated into media containing vitamins in the third test.

Results

The results of this series of experiments are given in Table 91. In this table the fungi are compared within the species rather than between the species since each species produced a different type of maximum growth. Amount of growth is coded numerically: 4 represents excellent and dense growth, 3 represents good growth, 2 represents moderate growth, 1 represents poor growth, the plus sign (+) indicates that some growth was visible, 0 represents no growth and the letters NT indicate that the fungus was not tested in a particular chemical. Results with the

Table 91. Growth of Fungi on various Vitamin, Nitrogen and Carbon Sources.

Nutrients	Fungi									
	<i>Aspergillus niger</i>	<i>Aspergillus glaucus</i>	<i>Geotrichum candidum</i>	<i>Phanerochaete chrysosporium</i>	<i>Penicillium lilacinum</i>	<i>Penicillium notatum</i>	<i>Penicillium chrysogenum</i>	<i>Aureobasidium pullulans</i>	<i>Trichoderma viride</i>	
Vitamins:										
Thiamine	+	4	4	4	4	4	4	4	4	4
Biotin	3	4	4	4	4	4	4	4	4	4
Thiamine + Biotin	3	4	4	4	4	4	4	4	4	4
Thiamine, Biotin, Inositol, + Pyridoxine	3	3	4	3	4	4	4	4	4	4
Nitrogen:										
1-Asparagine (NH ₄) ₂ SO ₄	4	4	4	4	4	4	4	4	4	4
NH ₄ NO ₃	2	4	3	4	3	N.T.	3	3	3	3
NaNO ₃	2	3	+	3	4	3	3	4	4	4
NaNO ₂	3	4	+	3	3	N.T.	3	3	3	3
Urea	3	3	4	3	3	N.T.	3	4	4	4
Carbon:										
Xylose	2	2	3	4	3	N.T.	3	4	3	3
Glucose	4	4	4	4	4	4	4	4	4	4
Mannose	4	3	4	4	4	N.T.	4	3	4	4
Fructose	2	4	4	4	4	N.T.	4	4	4	4
Sucrose	4	4	2	4	4	4	4	4	2	2
Lactose	1	4	+	+	+	+	3	1	3	3
Soluble Starch	4	4	+	1	3	3	3	1	2	2
Other:										
Amyl acetate	1	1	1	1	1	1	1	+	1	1
Tannic Acid	+	2	+	+	+	+	3	4	+	+
Phenol	0	0	0	0	0	0	0	0	0	0
Indulin A	1	1	1	1	1	+	1	1	1	1

Code: N.T. - Not Tested
+ - Trace amount of growth.

1 - Small amount of growth.
2 - Fair amount of growth.

3 - Good growth.
4 - Maximum amount of growth.

different types of nutrients studied will be described for each nutrient-type studied, and for each fungus studied.

Vitamins

The vitamins assayed in these tests included thiamine, biotin, inositol and pyridoxine. In addition to being tested separately, thiamine and biotin were assayed together and in the same medium with the other two. Fusarium aquaeductum was found to be deficient (at least in the strain tested) for thiamine, and Aureobasidium pullulans for biotin. Otherwise good growth was obtained in all cases tested. Sometimes when thiamine and biotin were combined, growth was depressed, as it was when all four substances were assayed in the same solution. Again, growth was speeded up. Dr. V. G. Lilly has indicated that when multiple vitamins are tested in an experiment under certain conditions growth is depressed to a certain extent.

Nitrogen

None of the fungi grew in the absence of nitrogen from the medium (Table 92). Thus none of these fungi can utilize atmospheric nitrogen. Geotrichum candidum did not grow in the presence of potassium nitrate or sodium nitrite as sole nitrogen sources. Growth of this fungus was very poor in the presence of ammonium nitrate as a sole nitrogen source. In the case of Fusarium aquaeductum, growth was moderately poor in the presence of ammonium sulphate or ammonium nitrate as sole nitrogen sources. Geotrichum candidum thus falls in Type III of the Robbins nitrogen utilization types, while the remainder fall in Type II.

Carbon

In the absence of organic carbon sources, growth in all cases was

weak. This indicated the presence of small amounts of contaminating substances, a certain amount of growth based on autolysis, and the inability of the fungi under consideration to utilize atmospheric or dissolved carbon dioxide as a carbon source.

Lactose was metabolized weakly or not at all by Fusarium aquaeductum, Geotrichum candidum, Penicillium lilacinum, P. melinii, and Phialophora jeanselmei. It was metabolized only moderately by Aureobasidium pullulans. Geotrichum candidum, Penicillium melinii and Aureobasidium pullulans grew weakly in the presence of soluble starch, while Trichoderma viride grew only moderately. All species tested grew equally well in glucose and sucrose except Geotrichum candidum which grew in sucrose only about half as well as in glucose, in contrast to strains studied by Bobrov (1951) which did not grow in sucrose. Glucose, xylose, fructose and mannose were about equally available to the fungi tested. Fusarium aquaeductum and Trichoderma viride grew most poorly on xylose and took longer to make their maximum growth on this carbohydrate. Levulose was not rapidly nor readily available to Fusarium aquaeductum.

In parallel with one set of carbon sources, cellulose was tested. Cellulose was made available as filter paper which had been chopped finely in the Waring Blendor. Approximately 80 mg chopped filter paper were added to each dilution bottle. Growth was observed in all cases especially by the middle or toward the end of the period of observation. Fusarium aquaeductum, Geotrichum candidum, and Phialophora jeanselmei showed no indication on a dry weight basis, that they were able to use cellulose in this form.

Hydrocarbons

Using the technique suggested by Reese, Cravetz and Mandels (1955), an attempt was made to compare the growth in shaken liquid culture of the same series of fungi with growth on glucose and on S. A. E. No. 20 motor oil. In the medium suggested, the sole carbon source on the one hand was 0.5 gm of glucose per 50 ml of solution, and on the other, 0.5 gm of motor oil. The solution and emulsion were autoclaved, pairs of flasks were inoculated with 1 ml of spore or cell suspension, and were shaken for 120 hr. at 150 oscillations per min. At the end of that time the material in the flasks was filtered through tared Gooch crucibles, oven dried and weighed. Table 93 compares the amount of dry weights obtained in the two types of media on the basis of the ratio of growth in oil to that in glucose. Ratios were calculated as averages of pairs of determinations.

Other carbon sources.

In separate studies using other carbon sources results were variable. Amyl acetate was tried in one series because excellent fungus growth had been observed along a stream carrying waste materials including this substance. Phenol, tannic acid and Indulin A were also employed as possible carbon source materials. In the case of Indulin A, if growth occurred it may have been on impurities or on small fractions of material split off during autoclaving. In addition to using these materials the basal medium was modified to exclude vitamins in one case and mineral salts in another. These three types of material were used in parallel for each of the four carbon sources - amyl acetate, phenol, tannic acid, and Indulin A. In general, less than half as much to as much growth was

Table 92. Nitrogen utilization

Fungus	N ₂	Nitrogen Source		
		NO ₃	NH ₄	Organic N only
<u>Fusarium aquaeductuum</u>	-	x	x	x
<u>Fusarium oxysporum</u>	-	x	x	x
<u>Geotrichum candidum</u>	-	-	x	x
<u>Penicillium lilacinum</u>	-	x	x	x
<u>Penicillium melinii</u>	-	x	x	x
<u>Penicillium ochrochloron</u>	-	x	x	x
<u>Aureobasidium pullulans</u>	-	x	x	x
<u>Phialophora jeanselmei</u>	-	x	x	x
<u>Trichoderma viride</u>	-	x	x	x

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Table 93. Growth of Fungi on Motor Oil

Fungus	Ratio of growth in oil to growth in glucose
<u>Fusarium aquaeductuum</u>	2.49
<u>Fusarium oxysporum</u>	.60
<u>Geotrichum candidum</u>	6.18
<u>Penicillium lilacinum</u>	7.58
<u>Penicillium melinii</u>	.73
<u>Penicillium ochrochloron</u>	.64
<u>Aureobasidium pullulans</u>	.51
<u>Phialophora jeanselmei</u>	3.96
<u>Trichoderma viride</u>	.68

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obtained in the vitamin-free medium as in the medium with vitamins. Very little, if any, growth was obtained in the absence of mineral salts.

In addition to the summary presented in Table 91a species-by-species analysis of nutrition requirements is appended. This will point up some of the variations and differences not readily observed in Table 91.

Discussion and Conclusions

In nature, a number of nutritional groups (Garrett, 1956) of fungi occur. All nutritional groups are not yet fully elucidated. It has not been ascertained whether a member of one nutritional group could become a member of another group in the absence or depletion of specific elements or compounds. Under some conditions, highly specialized groups of fungi have developed in which the species have become dependent upon other organisms for particular nutrient factors. Thus the fungus of the fungus-alga association called a lichen cannot produce its own vitamin supply but is totally dependent on the alga for its vitamin nutrition. Parasitic fungi, whether they are pathogens of plants, animals, or man, have become dependent, at least in their parasitic stages, for various nutrients including carbohydrates and nitrogen.

It has been noted that only 2 of the 9 species of fungi tested are deficient for part of their vitamin requirements. Fusarium aqueductum can grow in the absence of thiamine when biotin is present, and the inverse is true for Aureobasidium pullulans. On the basis of growth on the sampled trickling filters, there is no evidence that these fungi are dependent on other organisms for the vitamins in which they are apparently deficient.

Most of the nitrogen and carbon sources required by the tested fungi

can be expected to be present in sewage and polluted water either as a natural part of the pollution or as degradation products resulting from the metabolic activities of other organisms acting on various substances. The fact that many fungi cannot metabolize nitrates, have difficulty in using ammonium salts, and cannot recover carbon from lactose does not imply that such substances, if present, cannot be broken down in passage through sewage treatment plants, or in streams, since other organisms present may be able to metabolize them. All the fungi tested have a wide range of ability to metabolize organic waste materials and thus contribute to the degradation of dissolved and settleable materials. The tested fungi, as well as others with different ranges of tolerance for pollutant materials, are thus contributing to the removal of such materials whether this occurs in a stream or in a sewage plant.

The results of the study reported above indicate that there is a definite group of filamentous fungi which can become acclimated to an aquatic environment. Such fungi, usually considered to be members of the natural populations of soils and decaying materials, and usually thought to be inactive under water logged conditions of such environments, have become naturally adapted to the highly specialized environment of the sewage treatment plant through ready availability of nutrients, and an apparently more favorable level of competition for that food supply. While such a population appears to have been developed by strain selection from soil inhabiting ancestors, there is no indication that the majority of species in the group could not return to an existence in the soil at any time, their place being taken by a strain quickly adapting itself to the aquatic environment from a life previously lived in the soil.

Appendix - Vitamin, Nitrogen, and Carbon Nutrition of Nine Common Sewage Fungi.

Fusarium aqueductum

Vitamins - Deficient for thiamine.

Nitrogen - Growth rather slow but good in asparagine, potassium nitrate, urea and sodium nitrite; growth poor in ammonium sulfate and ammonium nitrate.

Carbon - Growth slow but good in glucose, sucrose, mannose and soluble starch; growth poor in xylose, levulose and lactose; growth weak in amyl acetate and Indulin A; no growth in phenol or tannic acid.

Fusarium oxysporum

Vitamins - Growth good on all media.

Nitrogen - Growth good on all substrata but best on asparagine.

Carbon - Growth good on all substrata but growth on lactose slower and on xylose not as good as on other carbon sources used. Weak concentrations of amyl acetate and Indulin A were used, but no growth was recorded on phenol. On tannic acid growth was not evident until after 10 days and the curve was still ascending when the experiment was discarded after 15 days.

Geotrichum candidum

Vitamins - Growth good and rapid on all media, the peak being reached in two days in contrast with other fungi whose growth peak was not reached for 5 to 7 days.

Nitrogen - Good growth on asparagin and urea; fair growth on ammonium sulfate and ammonium nitrate; no growth on potassium nitrate and sodium nitrite.

Carbon - Good growth on glucose, xylose, mannose, and levulose; poor growth on sucrose; no growth on lactose and soluble starch. Fair growth was obtained on amyl acetate and Indulin A, but no growth on phenol or tannic acid.

Phialophora jeanselmei

Vitamins - All vitamins tested were used with about equal vigor but peak growth was not obtained until after 9 to 12 days.

Nitrogen - Asparagine and ammonium sulfate were metabolized most vigorously but growth was good on all nitrogen sources tested. Again, peaks were not obtained until 9 to 12 days of incubation.

Carbon - No growth occurred in lactose; growth was weak in soluble starch; and good growth was obtained on other carbon sources after 7 to 9 days. Amyl acetate gave fair growth, Indulin A weak growth, and phenol and tannic acid, no growth.

Penicillium lilacinum

Vitamins - Good growth on all media, the peak being reached in 5 days.

Nitrogen - Good growth on all nitrogen sources used. Growth was weakest on ammonium sulfate and ammonium nitrate, best on asparagine.

Carbon - Good growth on all carbon sources tested except lactose. It should be noted that no significant growth on lactose was recorded during the first 12 days of incubation but that on the 14th day as much growth was recorded for this fungus as for its growth on any of the other carbon sources at the end of five days. Fair growth was noted on amyl acetate and Indulin A but no growth was recorded on phenol or tannic acid.

Penicillium melinii

Vitamins - Growth was good on all vitamins and combinations tested.

Nitrogen - Asparagine and ammonium nitrate gave good growth although the latter was used more slowly; sodium nitrite was used slowly and gave fair growth at the end of 14 days.

Carbon - Lactose and xylose were not used appreciably. Good growth was obtained on the other carbon sources used. Growth was fair on amyl acetate, very little occurred on Indulin A, and none on phenol or tannic acid.

Penicillium ochro-chloron

Vitamins - On thiamine and biotin used separately, growth peaks occurred at the end of 5 days. When these two vitamins were used together, and when pyridoxine and inositol were added, the peaks were slightly higher and occurred at the end of only two days.

Nitrogen - Growth on all substrata was good but growth was best on asparagine.

Carbon - All substrates gave good growth but the culture on lactose required seven days to reach a peak while those on the other carbon sources reached a peak in 5 days. Growth was weakest on soluble starch but the peak was not greatly different from that of those on other media. Fair growth was obtained on amyl acetate and Indulin A, and good growth was recorded on tannic acid. No growth occurred on phenol.

Aureobasidium pullulans

Vitamins - Deficient for biotin but growth good on other substrata.

Nitrogen - Growth was not as luxuriant as in the other species tested, but this may be attributable to its habit of forming a slimy pellicle on the surface of the medium rather than a floccose mass of aerial mycelium in addition to the loosely intertangled hyphae in the medium. Growth was

good on all media but poorest on ammonium sulfate.

Carbon - Growth fair on most media but poor on lactose and soluble starch. A slight amount of growth was recorded on amyl acetate and Indulin A; no growth was found on phenol, but growth on tannic acid was excellent.

Trichoderma viride

Vitamins - Good growth was obtained on all substrata.

Nitrogen - Good growth was obtained on all substrata. Asparagine yielded the best growth, while it took longer to reach a lower peak on ammonium sulfate.

Carbon - Xylose, sucrose and soluble starch yielded only fair growth while growth on other carbon sources was good. Amyl acetate and Indulin A showed fair amounts of growth but tannic acid and phenol were not used by this fungus.

C. Activity of Cellulose-Decomposing Fungi Isolated from Sewage-Polluted Water*

Sewage polluted waters contain spores, other disseminules, and actively growing thalli of fungi capable of cellulose degradation. Maki (1954) has shown that a large amount of cellulose is present in sewage, and that cellulose, both from this source and from such natural sources of pollution as decaying vegetation, is present in polluted waters. In general, cellulose deterioration in sewage is attributed to anaerobic cellulose-decomposing organisms (bacteria) in digesters. To determine whether or not fungi could attack cellulose in aerobic pure cultures and in competition with sewage organisms, experiments were devised in which strains of species of common molds were used both as spore inoculum and as mycelial inoculum in stationary liquid culture tests. Cotton cloth was used as the source of cellulose.

* Reproduced with permission from Sewage and Industrial Wastes 29:210-217, 1957.

Experimental Technique

Two sets of studies were made. In the first study, the activities of 10 species of fungi, isolated infrequently to very commonly from sewage-polluted water, were compared singly and in four different mixtures. Specially prepared suspensions of mycelium and spores of 9 common sewage fungi were used in the second set of studies.

First Study Series

Cotton duck cloth was cut into appropriate lengths and stripped so that the tested woven cloth measured 1 by 3 inches not counting raveled threads on each of the four sides. The solution in which the cloth was incubated with the spore or cell suspension was a carbon-free medium of the following composition:

Item	Quantity (ml)
Yeast extract (Difco) (1%)	10
NH_4NO_3 (10%)	10
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (10%)	3
KH_2PO_4 (0.1M)	10
NaOH (0.1M) to give pH 6.1-6.3	2 to 4
Distilled water	1,000

Additions of 10 ml of this solution were made to a 150 x 15 mm. culture tube in which a cloth strip had been placed. Following autoclaving, 1 ml. of spore or mycelial suspension from culture slants was pipetted onto the cloth, an aluminum cap was placed on the tube. The prepared tubes were stored in an incubator at 30°C for 6, 9 and 14 days. Five strips were removed at the end of each period for each fungus, washed, soaked 20-30 min. in 50% ethanol, rinsed in water, and dried under moderate weight between pieces of absorbent material to prevent excessive curling. At the completion of the experiment, these strips, together with

15 uninoculated control strips from each period, were tested for tensility on a Scott tensiometer. Results of the breaks were reported in pounds of tensile strength remaining in the strips. (Cloth strips were supplied by, and breaking strength tests were conducted by, Dr. E. T. Reese, Pioneering Research Laboratories, U.S. Army Natick Laboratories, Natick, Mass.)

Table 94 lists the species of fungi used in both the pure and mixed cultures in the first test, their frequency of occurrence in polluted water samples, and the approximate numbers of spores or mycelial fragments per ml of inoculum. The mixtures were chosen on the basis of their known inability to degrade cellulosic materials (White et al, 1948), or as combinations of commonly isolated species.

Second Study Series

In the second set of studies, two types of inoculum were used. The first type was a mycelium suspension developed by shaker culture (as described in the preceding section) and the second was a spore suspension developed on slants. For the spore suspensions used in both the first and second series of experiments, stock culture slants were prepared on phytone-dextrose agar slants, and the spores were scraped into 10 ml of sterile distilled water by rubbing with a pipette. Spores from 4 slants were blended in a total of 100 ml sterile distilled water and used immediately.

Test procedures were identical with those used in the first series of experiments. Incubation periods of 6, 12 and 18 days were used. In addition to using the test medium described above, 24-hour settled domestic sewage, and sterilized sewage were used as incubation media.

Table 94. Effect of fungi on the tensile strength of cotton duck strips.

Fungus	Natural Occurrence	Spores per ml of inoculum	Loss of tensile strength Days of incubation (%)	6 Days	9 Days	12 Days
No. Species						
1 <i>Trichoderma viride</i>	Very Common	45,000,000	88.3	60.3		93.0
2 <i>Aspergillus fumigatus</i>	Infrequent	5,000*	89.6	74.3		94.0
3 <i>Glomastix convoluta</i>	Infrequent	250,000	2.6	0		10.3#
4 <i>Penicillium lilacinum</i>	Very Common	550,000	0.7	0		1.9#
5 <i>Penicillium ochro-chloron</i>	Very Common	3,000,000	73.1	44.9		84.4
6 <i>Aspergillus fumigatus</i>	Common	5,000,000	80.5	58.6		92.8
7 <i>Fusarium equisetinum</i>	Very Common	150,000	0	17.3		72.8**
8 <i>Cladosporium cladosporioides</i>	Common	250,000	1.6	3.8		0 #
9 <i>Geotrichum candidum</i>	Very Common	17,600,000	1.4	0		2.6#
10 <i>Aureobasidium pullulans</i>	Very Common	12,000,000	3.7	0		0 #
MIXTURES						
11 4, 7, 9, 10	-	8,000,000	0	0	0	6.7#
12 4, 5, 7, 9	-	2,800,000	0	0	2.1	0 #
13 1, 4, 5, 6, 7, 9	-	8,000,000	46.5	46.5	63.1	77.5
14 All Species	-	4,000,000	56.8	56.8	82.5	87.1

*Few spores produced, culture and inoculum mostly mycelial.

#According to previous work, values less than 15 per cent are of little significance.

**Values include strips contaminated with *Aspergillus fumigatus*.

Settled domestic sewage could yield organisms which would compete with the fungi for the cellulose, whereas sterilized sewage could supply an added carbon source or nutrients which might speed up the cellulolytic activity of at least some strains of fungi used in the test. Test data are reported in Table 95.

Two series of fungal species were chosen for study. In the first set of experiments, a miscellaneous group of species was chosen, strains of which were known to be cellulolytic. Certain commonly isolated species not previously shown to have any effect on cellulose were also included. In the second study, the species of fungi most commonly isolated from sewage-polluted water were used.

Results

The results of the first series of tests, under the conditions of the experiment, are given in Table 94. The ability of these fungi to degrade cellulosic materials, as shown by these tensile strength studies, differs from some of the results obtained by White et al. (1948).

Trichoderma viride, Myrothecium verrucaria, Aspergillus fumigatus and Penicillium ochro-chloron were very active, while the remaining species were either inactive or only slightly active. The strains of Gliomastix convoluta, Cladosporium cladosporioides, and Geotrichum candidum used showed no significant activity, although strains reported by White et al (1948) and by Siu (1951) showed considerable activity. The inability of Pullularia pullulans to use cellulose is in agreement with the findings reported by White et al. (1948). Penicillium lilacinum is listed by Siu (1951) as being noncellulolytic, while Fusarium aquaeductuum and Penicillium ochro-chloron had not been tested previously for such activity.

Table 95. Comparative effect of mycelium and spore inoculum on tensile strength of Cotton Duck strips in Three Media.

Fungus	No. Species	Cells per ml inoculum		Remaining tensile strength / days of incubation (%)					
				6 days		12 days		18 days	
				Mycel.	Spores	Mycel.	Spores	Mycel.	Spores
				(a) Raw sewage settled 24 hrs. at 20°C.					
0	Control	-	-	100.0	100.0	101.9	112.4	97.0	104.2
1	<i>Fusarium aqueductum</i>	840,000	150,000	100.7	103.0	101.1	102.4	98.0	115.3
2	<i>Fusarium oxysporum</i>	800,000	125,000	94.1	91.8	53.6	96.0	65.1	98.9
3	<i>Geotrichum candidum</i>	980,000	18,000,000	102.4	78.8	103.6	114.6	105.6	105.7
4	<i>Penicillium lilacinum</i>	760,000	550,000	100.7	101.8	101.8	112.8	101.9	118.8
5	<i>Penicillium ochro-chloron</i>	1,600,000	3,000,000	96.9	85.5	98.0	105.2	100.6	102.3
6	<i>Aureobasidium pullulans</i>	-	12,000,000	-	96.1	-	116.2	-	109.5
7	<i>Trichoderma viride</i>	4,000,000	45,000,000	99.1	101.8	102.0	116.4	100.9	112.2
8	<i>Penicillium melinii</i>	4,000,000	5,000,000	98.3	93.7	94.0	104.2	101.1	100.2
9	<i>Phialophora jeanselmei</i>	1,400,000	1,500,000	100.2	104.0	96.7	99.7	101.0	109.3
10	Average	-	-	99.1	95.2	93.9	106.6	96.8	108.1
(b) Raw sewage settled 24 hrs. at 20°C.									
0	Control	-	-	84.4	89.4	54.9	29.2	36.9	8.7
1	<i>Fusarium aqueductum</i>	-	-	91.9	74.0	56.5	15.6	31.2	13.4
2	<i>Fusarium oxysporum</i>	-	-	82.9	90.1	48.7	17.8	42.1	13.0
3	<i>Geotrichum candidum</i>	-	-	93.6	80.0	62.7	1.7	41.4	14.5
4	<i>Penicillium lilacinum</i>	-	-	93.4	78.3	63.7	29.2	35.9	12.7
5	<i>Penicillium ochro-chloron</i>	-	-	91.0	91.8	62.7	4.5	43.4	15.3
6	<i>Aureobasidium pullulans</i>	-	-	-	64.9	-	15.1	-	11.6
7	<i>Trichoderma viride</i>	-	-	88.3	71.0	61.0	13.4	43.8	10.3
8	<i>Penicillium melinii</i>	-	-	87.2	73.6	57.9	15.4	34.3	3.7
9	<i>Phialophora jeanselmei</i>	-	-	92.7	72.7	69.4	12.1	47.2	5.4
10	Average	-	-	90.1	77.4	60.3	15.0	35.5	11.1
(c) Raw Sewage autoclaved 20 Min at 15 P.S.I.									
0	Control	-	-	102.1	107.9	100.2	98.7	102.7	112.4
1	<i>Fusarium aqueductum</i>	-	-	98.9	103.8	94.1	111.4	101.1	109.8
2	<i>Fusarium oxysporum</i>	-	-	95.9	96.4	87.5	95.9	85.5	106.3
3	<i>Geotrichum candidum</i>	-	-	106.1	93.7	101.9	113.4	98.4	122.1
4	<i>Penicillium lilacinum</i>	-	-	102.5	101.3	104.5	105.3	102.5	109.0
5	<i>Penicillium ochro-chloron</i>	-	-	96.1	101.4	101.1	104.5	94.5	109.9
6	<i>Aureobasidium pullulans</i>	-	-	-	100.4	-	102.8	-	110.5
7	<i>Trichoderma viride</i>	-	-	94.1	100.4	96.9	106.7	94.8	105.2
8	<i>Penicillium melinii</i>	-	-	92.8	102.1	95.3	88.2	92.8	87.7
9	<i>Phialophora jeanselmei</i>	-	-	98.5	100.4	97.4	104.2	91.8	103.2
10	Average	-	-	98.1	100.0	97.3	103.6	95.2	107.1

Fungus combinations

The use of mixed cultures in testing cellulolytic activity apparently has been confined to fungal cultures whose activities are known (Siu, 1951; Military Specification (1956). In selecting a mixture of species for the first study, it was thought that a combination of fungi found in certain polluted habitats might also give a positive reaction. Combinations 11 and 12 (Table 94), in which four of the more common species isolated from sewage were mixed, did not degrade cellulose. Apparently, in the presence of one or more of the three noncellulolytic species used, the potential cellulolytic activity of Penicillium ochro-chloron could not be expressed. However, when two additional cellulolytic species were used as in Combination 13 (Table 94), the three noncellulolytic species did not exert this suppression. When all species were mixed together, as in Combination 14 (Table 94), little suppression was exhibited, although the total activity was less than that exerted by any of the three cellulolytic species when used alone. Geotrichum candidum, included in all mixtures and cited by White et al. (1948) as Oospora lactis, is a highly variable species. Because of its great variability, including strains not known to be cellulolytic, its activity cannot be considered suppressed in Combinations 11 and 12.

The Second Study

The second study was made with seven species of fungi commonly isolated from sewage and polluted water (Nos. 1, 3, 4, 5, 6, 7, 9; Table 95). One species of occasional occurrence but of potential interest because of its ability to utilize hydrocarbons as a sole carbon source (No. 2, Table 95),

and another species isolated repeatedly from streams carrying acid coal mine wastes (No. 8, Table 95) were used without regard to previous assays. There is a possibility that one strain of a species may have different physiological requirements or activities than another. Two of the species under observation have been tested repeatedly by White et al. (1948), the others only once, if at all. In reporting the results, only the groups of breaks averaging more than 15 percent loss in tensile strength over the controls exposed for 6 days in the test medium were considered, although some breaks showing less than 15% loss were found to be significant within the limits of these experiments.

Inocula

Preparation of inocula was independent of cellulose conditioning. In little, if any, of the work reported by White et al. (1948) and by Reese and Downing (1951), is there mention of developing inocula using cellulose as a component of a culture medium designed to increase the yield of cellulase. Rather, it was emphasized that stock culture medium was potato dextrose agar with 2% dextrose. In the first of the studies in the second group only a mycelium inoculum was used (Table 95). Spores were present only in the case of those species which were able to produce spores in liquid shaken culture. This capacity was most apparent in the cases of Geotrichum candidum and Phialophora jeanselmei. In only one test with the test medium and in one with the sterilized sewage did much cellulolytic activity take place. Fusarium oxysporum reduced the tensile strength of the cloth to 65.1 and 85.5% of the control after 18 days of incubation in the test medium and sterilized sewage respectively. On the average, the tensile strength of the cloth was reduced only 4-5% by

mycelial suspension inoculum after 18 days of incubation.

Siu (1951) reported that when mycelial pads are used for inocula the activity rate of a test fungus in the presence of a fungicide is more rapid than when spores are introduced["] as an inoculum. This may be caused by the inhibitory effect of the fungicide on spore germination, whereas the mycelium in the pad is already very active. On the other hand, if a young mycelium is washed and stored in a neutral solution it is possible that it is not producing sufficient enzymes to be effective in a stationary liquid culture. (Rolison & Lumb, 1953).

In the test of spores in the second study (Table 95), a rather heavy spore suspension was prepared as inoculum. It was expected that at least Trichoderma viride and Penicillium ochro-chloron would reproduce the earlier results, but in both the test medium and the sterile sewage there was an increase in tensile strength of the cloth during the 18-day incubation period.

There was a marked reduction in tensile strength of the cloth, using raw sewage which had settled 24 hr. in a 20°C constant temperature chamber. The strength was reduced slightly less when mycelial suspensions rather than spore suspensions were used. The contents of raw sewage, of which samples were obtained several months apart for the two tests, were extremely variable from day to day and from hour to hour. However, this did not seem to account for the greater loss of tensile strength when spore inocula were used, although some fungal species are known not to degrade cellulose. It is possible that none of the fungi inoculated on the strips of cloth had cellulolytic effect, since organisms which carry on degradation may have been present in the raw sewage.

Statistical Analysis

A statistical analysis was made of the data in the second study.

The experiment was treated as a factorial design with four factors:

1. Length of treatment or "time" (T) at three levels: 6, 12, and 18 days.
2. Inoculum (I) of two types, mycelium and spores.
3. Medium (Mm), of three types: mineral salts, raw sewage, and sterilized sewage.
4. Mold (Md) of which there were 9 species, one control and 8 experimental. Mold 6, Table 95, was not included in these analyses.

There existed $3 \times 2 \times 3 \times 9$ or 162 combinations of the various levels of these factors, 144 of which were replicated 5 times and the remaining 18 were replicated 15 times. There were 990 individual measurements taken. The variable used was pounds of tensile strength required to break the test strips as measured with a Scott tensilometer.

Results of a preliminary four-factor analysis of the data involving the eight fungi, exclusive of the controls, are presented in Table 96. The computations of the variance components were performed by the usual methods for a complete factorial experiment. The large medium main effect was caused by the breaking strength values for strips exposed in the raw sewage medium which were about one-half the values obtained from strips exposed in the other two media.

A three-factor analysis was then made for each of the three media used, but it was found that for a given medium the magnitude of the difference

Table 96. Four-Factor Experiment, Controls omitted.

No.	Source of Variation	Degrees of Freedom	Estimate of Variance	"F" Ratio
1	Medium (Mn)	2	705,133	(1)/(6) = 7.18*
2	Mold (Md)	7	4,616	(2)/(9) = 2.37
3	Time (T)	2	68,619	(3)/(6) = .70
4	Inoculum (I)	1	20,775	(4)/(19) = .77
5	Mn X Md	14	1,698	(5)/(17) = 2.98#
6	Mn X T	4	98,266	(6)/(14) = 8.13*
7	Mn X I	2	81,391	(7)/(14) = 6.73
8	Md X T	14	1,802	(8)/(17) = 1.41
9	Md X I	7	1,944	(9)/(17) = 3.41#
10	T X I	2	2,364	(10)/(14) = .39
11	Mn X Md X T	28	523	(11)/(15) = 1.06
12	Mn X Md X I	14	786	(12)/(15) = 1.60
13	Md X T X I	14	607	(13)/(15) = 1.24
14	Mn X T X I	4	12,093	(14)/(15) = 24.60#
15	Mn X Md X T X I	28	492	(15)/(16) = 1.59*
16	Residual	567	309	-
17	Pool (11), (12), (13), and (15).	84	570	-
18	Pool (17) and (8).	98	603	-
19	Pool (14), (7) and (10).	8	26,985	-

* Significant at 5 per cent probability level.

Significant at 1 per cent probability level.

between inocula depended on the length of treatment. Therefore, two-factor (I and Md) analyses were made for each length of treatment for each medium.

Three types of results were obtained from these two-factor analyses. Of these, the most important result was that there was no interaction between inoculum and mold. That is, the difference between inocula was the same for each mold tested for a given length of treatment. Each of the three raw sewage experiments gave similar results. The strips from the spore inoculum had one-fourth to one-fifth the breaking strength of those from the mycelial inoculum after 18 days of incubation.

In comparing a mold with its control strips for both kinds of inocula, and for a given length of treatment and given medium, eight types of results were found, most of which were based on rather minor variations in the overall picture of significant cellulolytic action by the experimental mold. Geotrichum candidum, Fusarium oxysporum and Penicillium (nr.) melinii gave some appreciable cellulolytic activity. In this analysis, values were based on pounds of tensile strength lost rather than on a comparison of percentage losses. In the test medium, spore inocula were more active after six days than mycelial inocula in the case of Geotrichum candidum, whereas mycelial inocula of Fusarium oxysporum were more active after 12 and 18 days of incubation. In raw sewage, combined values for spore inocula of all the molds used gave lower values at each harvest period than mycelial inocula. In sterilized sewage at the end of six days, a mycelial inoculum of Geotrichum candidum showed some activity, whereas at the end of 18 days a mycelial inoculum of Fusarium oxysporum and a spore inoculum of Penicillium (nr.) melinii showed appreciable increases in activity.

Six two-factor analyses, comparing lengths of treatment and different molds for a given medium and a given inoculum, were studied so that changes in breaking strength with variation in incubation time could be observed. No changes significant for demonstrating cellulolytic activity of fungi were found.

Discussion

In sewage-polluted waters such as those from which the fungi under consideration were obtained, there may be many carbohydrates present. Some of these may be simple pentoses or hexoses which would be degradation products of sewage solids not available for use by the organisms through whose actions they were produced. Maki (1954) has shown that glucose is a metabolic byproduct formed by one group of cellulolytic bacteria which cannot use it in its metabolism. While such substances may be present in dilute solution, they are usually in sufficient quantity to help in maintaining a population of microorganisms capable of using them as food. Such microorganisms include those filamentous fungi under consideration in this study.

Certain fungi have been shown (White et al. 1948) to be able to use cellulose at least in pure culture. In one series of experiments, strains of fungi found more commonly in sewage-polluted waters were shown to be able to use cellulose in pure culture. It seems possible, then, that these fungi may be able to use cellulose as a food substrate in sewage-polluted waters where cellulose is present in large amounts.

When the more common species of fungi isolated from sewage-polluted waters are tested in mixed fungal cultures, the cellulolytic activity of certain species appears to be reduced. Mixtures of species containing

certain strains known not to use cellulose, give no loss of tensile strength of test cloth strips, while mixtures containing strains of species known to use cellulose showed less loss of tensile strength than when the known users were tested in pure culture.

Following a test of 9 commonly occurring sewage fungi on cotton cloth, it was found that in a mineral salts solution little activity occurred except for strains of two species known to be cellulolytic. There was no increase in activity when sterilized sewage was used. In the case of raw sewage, the amount of cellulolytic activity in general was correlated with the sewage rather than the fungi since there was no significant difference between the species of fungi used. Because washed mycelial inoculum was used, it was possible that insufficient amounts of cellulase were being produced by this type of inoculum. In a second test, spore suspensions rather than mycelial suspensions were used. The purpose of the test was to determine whether germinating spores and young mycelium, which had not previously been washed, would be better producers of cellulase than washed mycelium. The principal difference between the two experiments was a uniformly better action of the spore inoculum in raw sewage. Except for a slightly increased tensility of the cloth in mineral salts and sterile sewage media, no other effects were noted.

Statistical analyses of the second half of this experiment gave little indication that one fungus was better than another in this type of activity.

Two possible explanations for this lack of cellulolytic activity are based on data from the second set of experiments. First, the cellulase producing capacity of the test organisms was reduced during two to three

years storage in the laboratory on media containing no more complex carbohydrates than glucose. Second, populations present in the sewage which could utilize cellulose were encouraged to increase through the release of byproducts of fungal inoculum activity and the removal of toxic materials by other organisms present in the complex populations.

D. BOD Satisfaction by Fungi *

Several fungi have been isolated in significant numbers from various locations in a sewage treatment plant and in a stream both above and below a sewage outfall. Available information (see above) shows that certain fungi occur regularly in such habitats and apparently are able to multiply therein. Melin and Nannfeldt (1934) found that fungi were able to increase in paper mill white water, and Ruchhoft, Ettinger and Walker (1940) found that mold-like growths occurred in biochemical oxygen demand (BOD) test bottles after 5 days incubation in highly acid waters (pH 2.7).

All fungi studied to date require free or dissolved oxygen for growth and reproduction. They are thus aerobic organisms and should be able to function and produce growth under the conditions described below. Certain fungi, including yeasts, are able to carry on fermentative functions without the presence of free or dissolved oxygen. They do this at the expense of preformed tissue.

The BOD determination was used as a tool in this investigation to evaluate the oxygen consuming capacity of certain species of fungi and sewage organisms, either alone or in combination. The results should indicate whether these fungi can readily oxidize organic materials and thereby successfully compete for nutrients in the natural purification of polluted waters.

The choice of molds to be used in the test was based on their prevalence in sewage and polluted water. Most species selected were found

*Reproduced with permission from Sewage and Industrial Wastes 28:1075-1086, 1956.

abundantly in the polluted waters of Lytle Creek, Clinton Co., Ohio; in various parts of the Dayton, Montgomery Co., Ohio, sewage treatment plant; and in the sewage treatment plant at Yellow Springs, Greene Co., Ohio. In addition, some of the species were found in isolations from a stream carrying wastes from a pharmaceutical plant at Lawrenceburg, Dearborn Co., Ind., and in samples from the Ohio River and from a local sewer.

Experimental Technique

Eight commonly isolated species of fungi were chosen for this study. They include Fusarium aquaeductuum, F. oxysporum, Geotrichum candidum, Phialophora jeanselmei, Penicillium lilacinum, P. nr. melinii, P. ochrochloron, and Trichoderma viride. The strain of F. oxysporum, isolated from a local sewer, showed a tendency to utilize hydrocarbons in dilution water, and the strain of P. nr. melinii was isolated from streams carrying acid mine wastes.

Mold Seed

Mold seed was prepared as follows: a needle-full of spores was transferred from a stock culture slant to 100 ml of sterile phytone-dextrose broth (pH approximately that of subsequent tests) in a 250 ml Erlenmeyer flask, shaken on a rotary shaker for three days at room temperature, filtered through a sterile membrane filter. The resulting mycelial mat was washed and floated off the filter into 100 ml sterile distilled water in another 250 ml flask. The mat was broken up in a Waring Blendor the jar of which had been autoclaved at 20 lb for 15 min., and the resulting suspension was stored in a refrigerator until needed. Such

suspensions have remained viable for as long as 5 months but in the present study they were used within 2 weeks. This type of inoculum was preferred to a simple spore suspension in which lag periods might have developed during spore germination.

Ten milliliters of the mold seed suspension were added to 100 ml of sterile distilled water, and 25 ml aliquots of the freshly prepared dilute suspension were used in seeding each daily experiment. Mold counts showed that 5,000 to 25,000 spores or apparently viable cells per ml of aliquot were added to the solutions described below to give 0.5% (by volume) of mold seed.

Sewage Samples

Sewage was collected from a sanitary sewer serving a residential area as soon after 8:30 A.M. as possible. Sewage was not collected on days when rains produced a heavy runoff of silted or clay-carrying water. Sewage which had settled 48 hr. was used in experiments at pH 2.9 and 5.1, and 24-hr. settled sewage was used in experiments at pH 7.2 and 9.5. In addition, for experiments at pH 2.9 a portion of the sewage was allowed to settle for about 6 hr., the pH was adjusted to 3.0, and settling continued for the remainder of the 48-hr. period. No count is available of the organisms in the sewages used, nor are there any available data on the types of organisms seeded into the experiments as "normal sewage seed".

Dilution Water

"Formula C" dilution water was prepared according to Standard Methods (1955). Buffers for pH 5.1 and 9.5 were made up according to Gortner and Gortner (1949). For pH 5.1, a Na_2HPO_4 - KH_2PO_4 solution was

prepared to approximate a theoretical pH of 5.0. After autoclaving this gave a pH of 5.1. For pH 9.5, a boric acid-KCl-NaOH mixture was prepared to give a theoretical pH of 10.0. After autoclaving the pH was 9.5 ± 0.2 . For pH 5.1 and 7.2 buffers, the CaCl_2 and MgSO_4 solutions had to be autoclaved separately to prevent precipitation. For approximate pH 3.0, 37.0 ml of a solution of 10 g / l concentrated H_2SO_4 was added to 5 l of dilution water. All pH measurements were made with a Beckman glass electrode pH meter.

Substrate

The synthetic substrate contained 150 p.p.m. each of glucose and glutamic acid in dilution water adjusted to the pH of the experiment. To compensate for evaporation during sterilization, 100 ml distilled water were added. All glassware, siphons, connectors, rubber stoppers, foil-capped B.O.D. bottles and solutions were sterilized in an autoclave at 20 lb pressure for 30 min.

Test Procedures

Following overnight storage at 20°C ., the bottles of adjusted dilution water were removed to the laboratory and seeded with the mold or sewage seeds. For pH levels of 5.1, 7.2, and 9.5, the seven 8 l serum bottles were seeded as shown in Table 97.

For studies at pH 2.9, it was necessary to add two additional series. These were inserted after bottles Nos. 2 and 5, and contained sewage seed acclimatized at pH 3.0 in the same amount as unacclimatized sewage seed. In bottles Nos. 4 and 7, pH 3.0 acclimatized sewage seed was used with the mold seed.

Table 97. Serum Bottle Additives and Seeds.

Bottle No.	Additives and Seed*
1	2.0 per cent glucose-glutamic acid.
2	0.6 per cent sewage seed.
3	0.5 per cent mold seed.
4	0.6 per cent sewage seed, 0.5 per cent mold seed.
5	2.0 per cent glucose glutamic acid, plus 0.3 per cent sewage seed.
6	2.0 per cent glucose-glutamic acid, plus 0.5 per cent mold seed.
7	2.0 per cent glucose-glutamic acid, plus 0.3 per cent sewage seed and 0.5 per cent mold seed.

* Includes 5 l. of dilution water.

After seeding the serum bottles, the cotton stoppers were replaced with sterile rubber stoppers, the bottles were shaken in order to raise the dissolved oxygen level as high as possible (usually between 7.0 and 8.5 p.p.m.), and portions of the contents were aseptically siphoned into 13 sterilized B.O.D. bottles. The remaining solution was used for pH determinations.

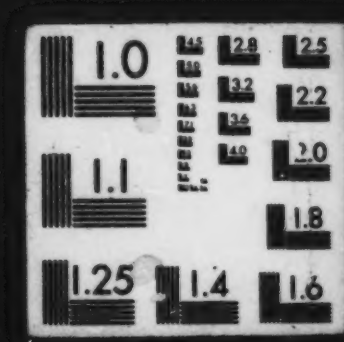
The first and last bottles siphoned were used for the determination of the initial dissolved oxygen. Ten B.O.D. bottles were stored by inversion in water baths at 20°C. One bottle was stored for a final test of the pH. The water baths in which bottles 1, 3 and 6 were incubated were dosed with 1 ml of an iodine-potassium iodide solution (prepared by adding 100 g of I_2 and 130 g of KI to 1 l of water). With each withdrawal of B.O.D. bottles for the D.O. determination (at 2-day intervals) an additional 0.5 ml. of the I_2 -KI solution was added to each tray. This solution prevented contamination of pure cultures by motile bacteria which could penetrate the water seal around the glass stoppers. Prior to each D.O. determination, 1 ml. of sample was taken from the bottle and plated in phytone-dextrose agar to determine the bacterial and fungus count. Where contamination occurred shortly after initiation of the incubation period, or where wide discrepancies in the D.O. determinations were found, the series at that particular pH was repeated.

For calculations of the k and L values, the method described by Moore et al (1950), was used. Duration of the lag period was determined by plotting the B.O.D. curve. After oxygen depletion was established, a tangent to the curve at the point of intersection with the abscissa at

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zero time was used to locate the initial time for rate calculations. The remaining observed B.O.D. values were used to calculate the rate (k) and first stage B.O.D. limit (L) on the appropriate graph for the moments method. Moments method curves for various time sequences have been drawn up by Kramer (1955), and these were used for interpolation of data and determination of k and L values.

Results

It was noted that in other experiments where more concentrated nutrients and larger amounts of inoculum are used, the unbuffered pH at the end of the experiment might be quite different from that at the beginning. In most cases in the present experiment, the pH levels remained constant throughout the 10-day incubation period. At pH levels 2.9 and 9.5, a lag period ranging from one to 4 days was noted. Where fungus growth occurred, the lag period was shorter than that for sewage seeds.

Average 2-, 4-, 6-, 8-, and 10-day B.O.D. values for the fungi tested at the several pH levels are presented in Table 98. Figures 49, 50, 51, 52 and 53 illustrate the minimum, average and maximum B.O.D. values of each type of inoculum for each pH level. In separate experiments, individual fungi demonstrated three types of growth rate. In a complete medium, with organic sources of nitrogen, and with major and minor elements and vitamins, Phialophora jeanselmei took 8 days to reach maximum development, while Geotrichum candidum took only 2 days, and the other species listed above took 3 or 4 days. In the suboptimum conditions for fungus growth in the B.O.D. bottle with only minimal amounts of macromineral elements, and

Table 98. Comparative B. O. D. of fungi in pure culture and various sewage seeds.

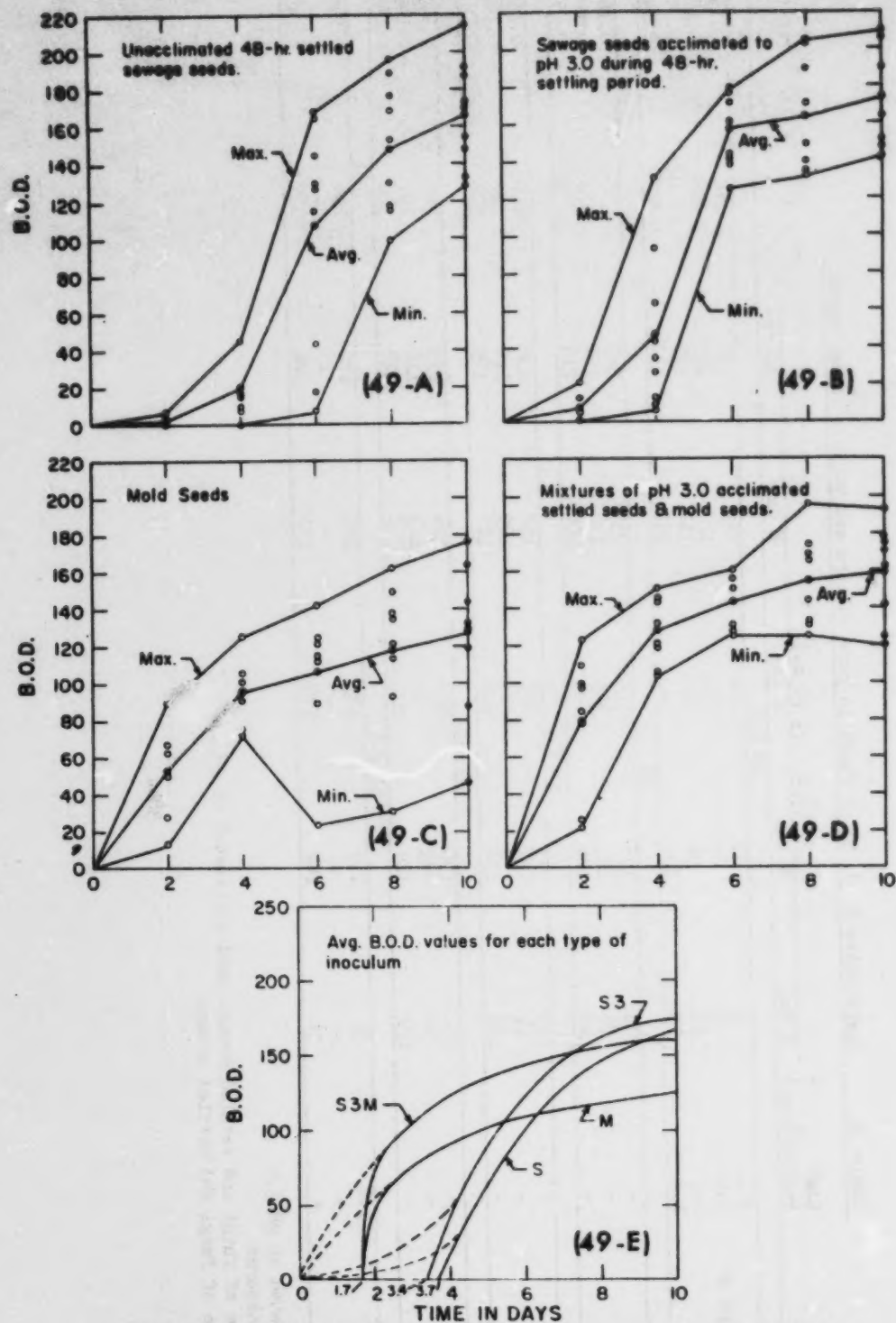
Seed	Lag Period(days)	B. O. D. (p. p. m.)					L	k
		2 day	4 day	6 day	8 day	10 day		
pH 2.9								
Settled sewage	3.7	3	20	108	149	167	201	0.154
Settled sewage, pH 3*	3.4	8	47	157	163	173	214	0.171
Fungi**	1.7	53	96	107	118	128	150	0.205
Mixture***	1.7	79	129	143	155	160	182	0.144
pH 5.1								
Settled Sewage	-	134	197	220	233	245	245	0.160
Fungi **	-	87	123	145	138	151	151	0.160
Mixture****	-	140	184	199	208	216	221	0.192
pH 6.7								
Settled Sewage	-	125	131	164	198	194	200	0.156
Fungi**	-	44	82	97	109	112	127	0.102
Mixture****	-	126	150	176	187	185	184	0.22
pH 7.2								
Settled Sewage	-	132	190	207	224	233	242	0.191
Fungi**	-	54	83	99	113	118	129	0.149
Mixture****	-	139	185	201	218	228	230	0.192
pH 9.5								
Settled Sewage	1.6	31	119	149	168	172	166	0.145
Fungi**	-	-	-	-	-	-	-	-
Mixture****	-	51	144	178	190	206	225	0.138

* Acclimated at pH 3.0

** Pure Cultures

*** Mixture of fungi and settled sewage seed acclimated at pH 3.

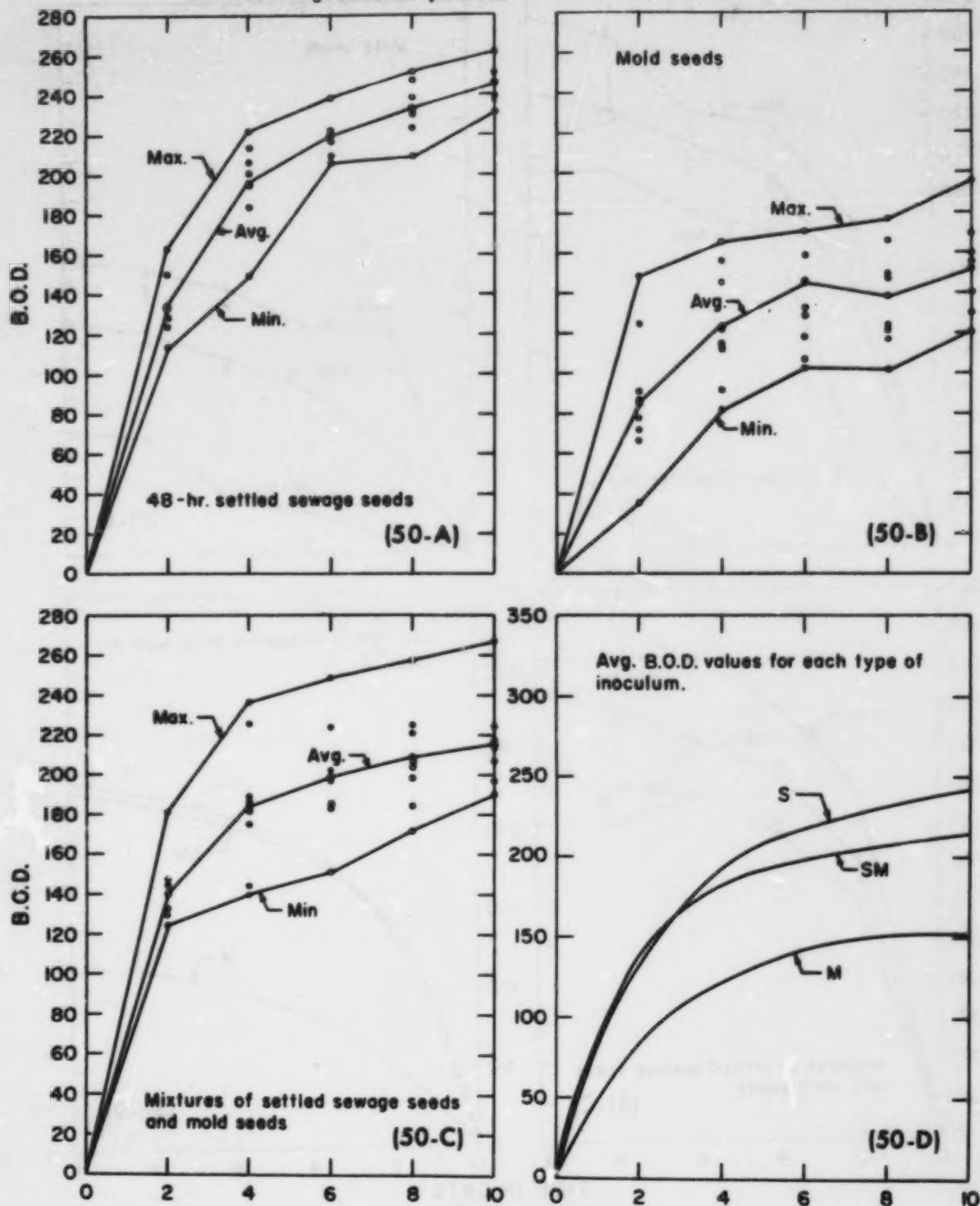
**** Mixture of fungi and settled sewage.



B.O.D. IN EXPERIMENTS WITH 9 MOLD SEEDS IN A DILUTE NUTRIENT SOLUTION ADJUSTED TO pH 2.9

FIGURE 49

BOD in experiments with 8 mold seeds in a dilute nutrient
solution adjusted to pH 5.1.

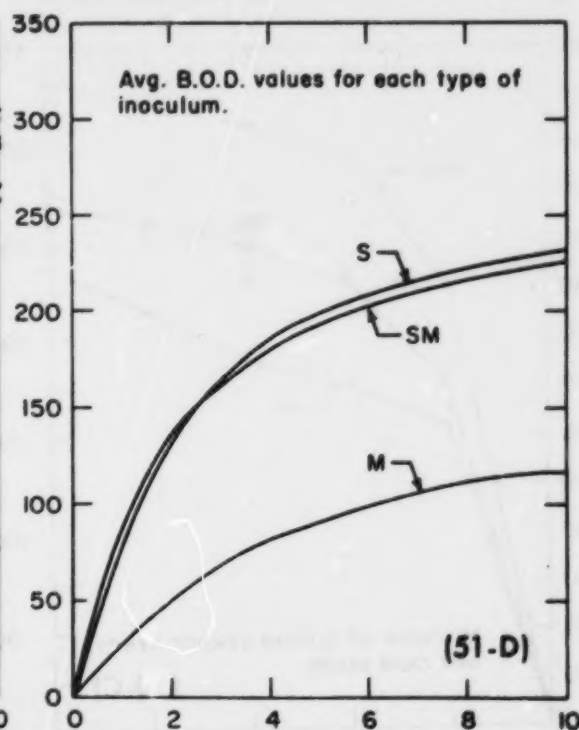
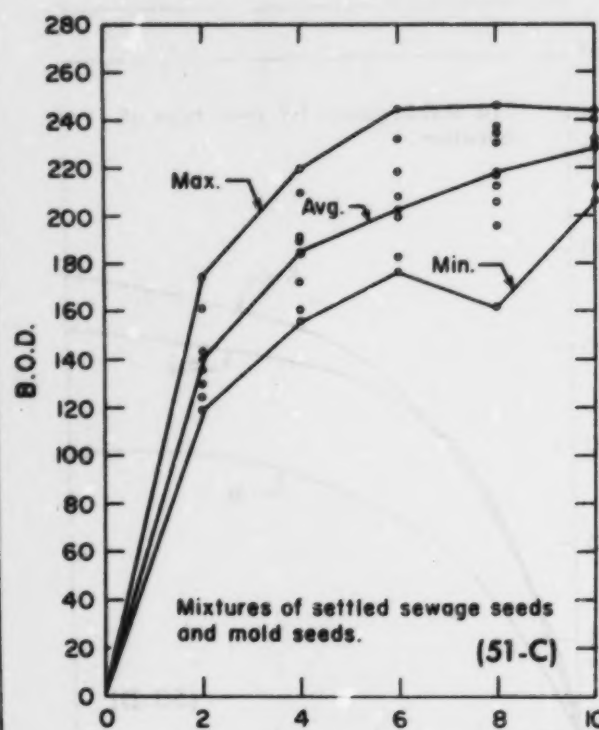
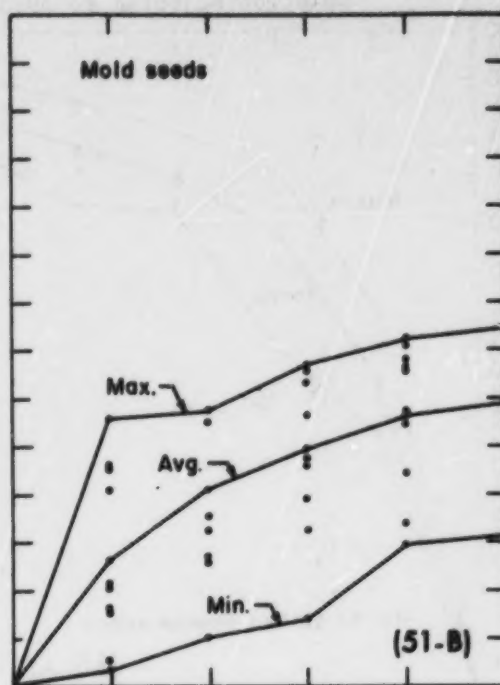
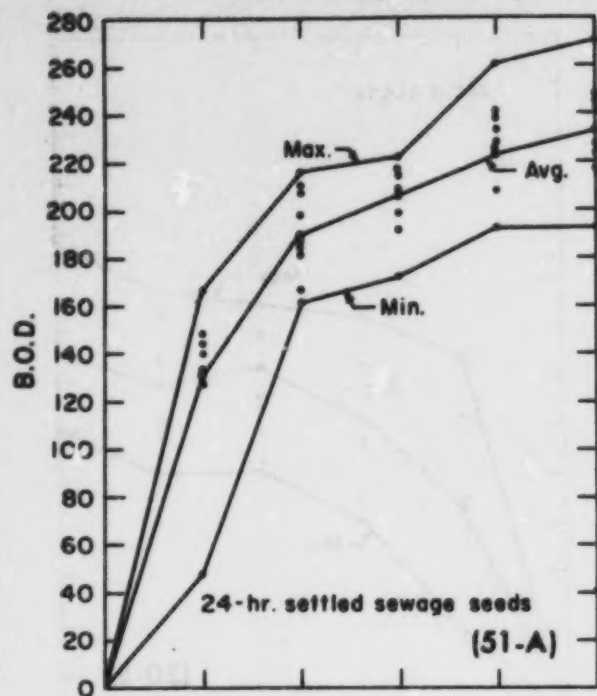


TIME IN DAYS

FIGURE 50

384c

BOD in experiments with 10 mold seeds in a dilute nutrient solution adjusted to pH 7.2.



TIME IN DAYS

FIGURE 51

BOD in an experiment with one mold seed in an unbuffered dilute nutrient solution (pH 6.5). S = sewage seed; M = mold seed; SM = mixed sewage and mold seed.

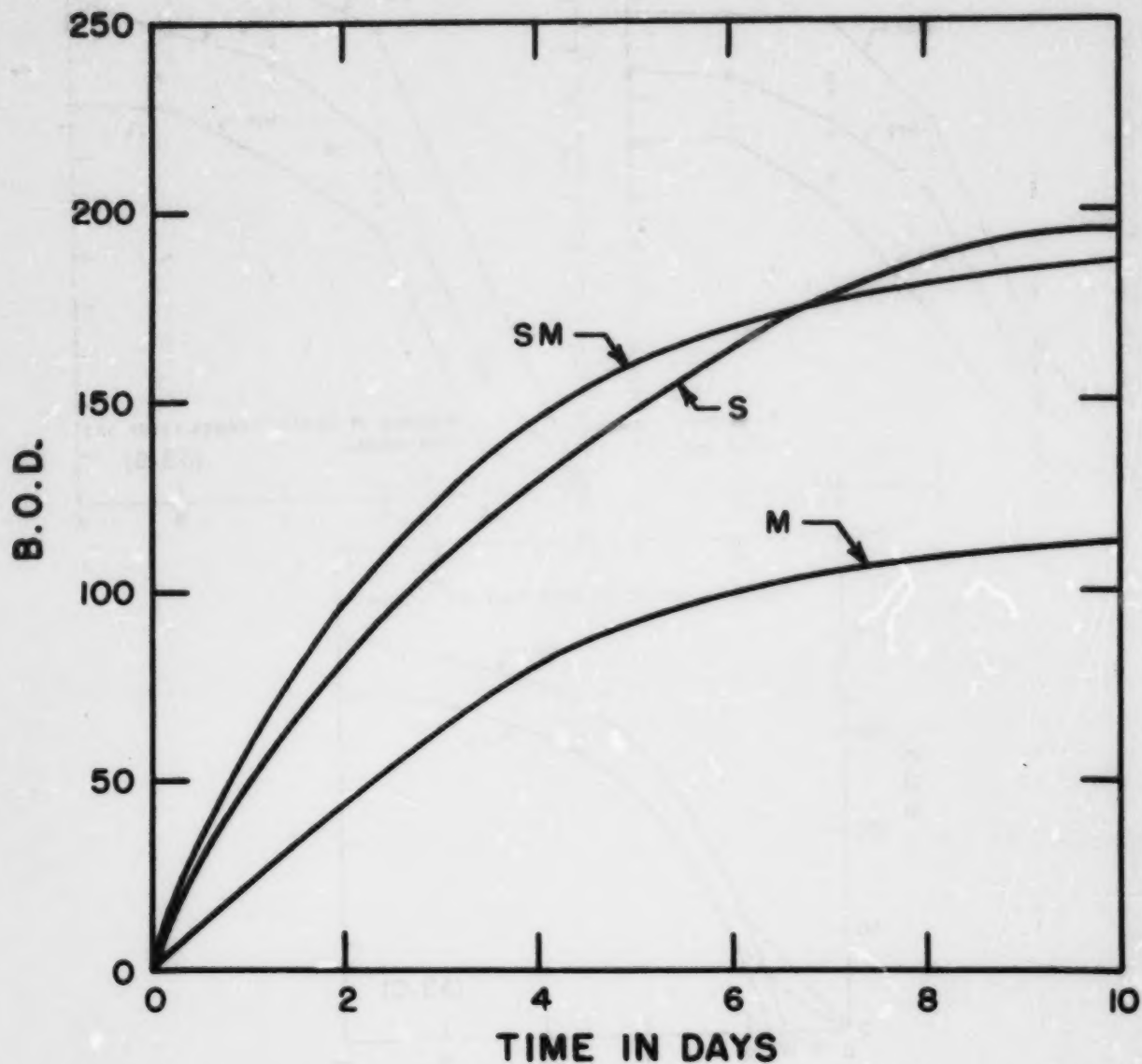
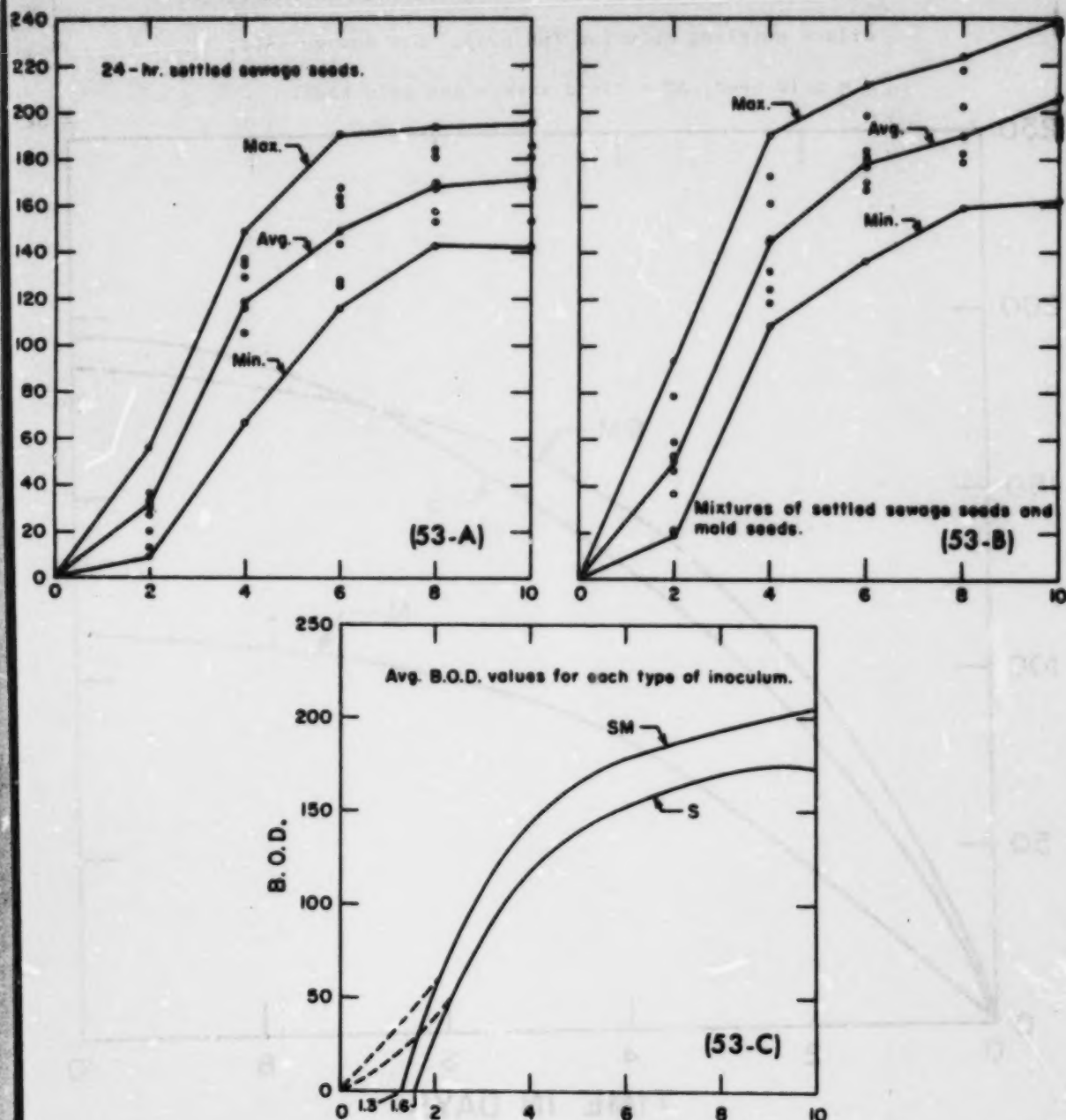


FIGURE 52

BOD in experiments with 8 mold seeds in a dilute nutrient solution adjusted to pH 9.5.



TIME IN DAYS
FIGURE 53

with only glucose and glutamic acid in weak solution as carbon and nitrogen sources, G. candidum never was as active in the use of dissolved oxygen as the other fungi.

The results for experiments at pH 2.9 are shown in Figure 49. The average for each of the four types of seedings at this pH is given in Table 98. Unadjusted sewage seed showed a slightly longer lag period than seed acclimated to pH 3.0 during settling. In one case this lag period was of such a length that k and L values could not be calculated by the moments method or any supplemental variation of it. Three mold seeds showed appreciable lag periods, but because of the B.O.D. values developed during the course of the incubation period the moments method could not be used without supplementation in calculating k and L values for 2 of these molds. Where mixed mold and sewage seeds were used, three mold species developed lag periods. Here, in two cases, moments method calculations gave unusually high, thus unsatisfactory, k and L values. Because fungi grow best at low pH levels, the more rapid use of dissolved oxygen in the initial periods of the incubation was expected. When mixed mold and sewage seeds were used, the more rapid utilization of dissolved oxygen was attributed partly to use by fungi of nutrients added in the sewage seed conditioned to a low pH.

At pH 5.1 and 7.2, sewage seed activity more nearly approximated that expected. These samples showed little difference in activity rates, and therefore no effort has been made to correlate these small fluctuations with date of collection or with types of domestic wastes such as laundry water in the sewage.

Mold seeds at pH 5.1 (Figure 50 and Table 98) showed greater activity than at pH 7.2 (Figure 51 and Table 98). At pH 5.1, activity of the fungi is approximately 0.4 to 0.6 that of sewage seed as indicated by the 10-day B.O.D. values, although the rates at which this activity is attained are quite variable. At pH 7.2, the amount of fungus activity, compared with that of sewage seed, was approximately 0.3 to 0.5 as indicated by 10-day B.O.D. values. Again G. candidum was less active than the other fungi tested. At pH 5.1, mixtures of fungus and sewage seeds were almost as active in removal of dissolved oxygen as was sewage seed alone. At pH 7.2, activity of mixed seeds was more erratic than at pH 5.1, since some produced higher 10-day B.O.D. values, others lower. In two cases, at pH 5.1, k and L values could not be computed using the moments method or supplements of it, because the values to be used in entering the charts were either too high or too low.

In one instance dilution water was unbuffered in setting up the experiment (Figure 52 and Table 98). Here the pH of the experiment was 6.5 in most cases. Except for slightly lower values for the activity of the mold seed as indicated by the 10-day B.O.D. value, there was little difference between this experiment and the series carried out at pH 7.2.

Sewage seed reacted more slowly at pH 9.5 (Figure 53 and Table 98) than at the other pH levels, but the time lag was less than 2 days. Mold seed produced in broth cultures at pH 9.0 and pH 6.0 showed no appreciable activity in B.O.D. development. On the other hand, when mold seed was added to sewage seed the resulting activity at pH 9.5 exceeded that of the sewage seed and approached the activity of mixed seed at pH 5.1 and pH 7.2. Even here there was a lag of 1 to 1.5 days before activity became evident.

To compute k and L values for this phase of the experiment, supplemental moment methods techniques were necessary.

Discussion

In the microbiology of sewage treatment and polluted waters, the roles of bacteria, protozoans and algae in the purification process have been emphasized. Until recently little attention has been paid to the activity of fungus populations of such habitats. Certain obvious species of fungi have been mentioned in the literature, but these have been considered more as nuisances than as beneficial contributors to purification processes.

Fungi require carbon and nitrogen, as well as a number of metallic ions in their metabolism. Because nitrogen is used in elaboration of protoplasm and cell wall material, its presence in a readily available form is critical for fungus growth. The B.O.D. test medium made up of dilution water, glucose and glutamic acid was designed to promote growth in dilute media of as many as possible of the organisms present in sewage. (The whole formula is considered to be an artificial sewage.) Minimal levels of nitrogen have been observed in few studies set up to determine the type of nitrogen used to best advantage by the fungi; the media used may have contained as much as 500 p.p.m. nitrogen or more. Stock culture media, based on the soy hydrolysate, phytone, in which at least 15 types of amino acids are present, has at least 490 p.p.m. nitrogen, as did the media in which the bulk inocula used in this experiment were produced. It is of interest, therefore, that in the B.O.D. bottles in which the seeded dilution water and nutrient medium solution was incubated, only 0.28 p.p.m. nitrogen

was present as the sole nitrogen source for the 125,000 to 625,000 mold cells used as inoculum. In all tests these cells remained viable and in most they produced growth.

When used alone in pure culture, mold seeds were able to start active use of dissolved oxygen within the first 2-day B.O.D. period at pH levels of 2.9, 5.1 and 7.2. The inactivity of molds at pH 9.5 indicates that the strains used were unable to grow alone under the conditions of the experiment or were inhibited by the concentration of boron in the buffer.

Of the fungal strains studied, only two were able to produce growth in nutrient broth at pH 9.0; these two were incapable of growth under the conditions of the B.O.D. experiment, but the organisms were still viable at the end of the 10-day incubation period, as shown by growth on agar plates. Except at pH 9.5, all pure mold cultures showed visible growth in the form of grey streamers in the presence of glucose and glutamic acid, and in all mixtures of mold and sewage seeds, growth became evident within two to four days of the initiation of the experiment and continued evident through the 10-day period. That B.O.D. at pH 9.5 was not attributable to B.O.D. exerted by dead tissues was indicated by growth of pure cultures of the inoculated organisms. The isolation of molds from white water samples indicates that certain species have become acclimated to conditions of high pH, or that conditions of high pH have not killed spores and other disseminules which were present normally or accidentally under such conditions.

Results of tests on mold cultures showed that the I_2 -KI solution added to the incubation trays at 2-day intervals effectively suppressed bacterial growth. On some occasions, bacteria developed in the B.O.D.

bottles at the end of 8 or 10 days, but in quantities small enough so that the results apparently were not affected by their presence. If bacterial growth of more than 100,000 colonies per milliliter was obtained it was considered to be contributing to the depletion of the dissolved oxygen. Demonstration of large numbers of bacteria in "pure culture mold seed" bottles called for repetition of the experiment. Mold growth, based on plating techniques was recorded as coded quantities. Many species of molds do not sporulate freely in liquid culture, so that when mold growth occurred as masses of mycelium in the B. O. D. bottles an occasional plate would appear to show no growth although visible growth was observed in the bottle. Plating of mold and bacterial samples further indicated the viability of the cultures being tested.

Several investigators have studied the ability of various microorganisms in pure culture and in combinations to oxidize organic materials. Butterfield and Purdy (1931) measured the oxygen depletion capacities of Bacterium aerogenes and the ciliated protozoan Colpidium sp. in pure cultures and in mixtures, and compared the findings with those of mixed bacterial cultures, of mixed bacterial cultures and Colpidium, and of normal biota (bacteria and plankton) in river water. They found that the Colpidium culture would not grow alone in a dilute medium but did grow well when 500 to 5,000 mg of dextrose and peptone were used per liter in the absence of bacteria. The culture of B. aerogenes gave an oxygen depletion of only 37.8% of that of "all bacteria and plankton in river water", while a mixed bacterial culture which was plankton-free gave 57.3%, a mixture of B. aerogenes and Colpidium gave 70.7%, and a mixed culture of bacteria with Colpidium gave 78.0%. Other pure cultures of bacteria gave results

similar to those for B. aerogenes, while other protozoans used in mixtures gave results similar to the mixtures of bacteria and Colpidium.

Sewage seeds alone were most active at pH 7.2 as was expected. No lag period was demonstrated at pH 5.1, and the amount and rate of activity were similar to those at pH 7.2. At pH 2.9, sewage seed demonstrated a lag of 2 to 4 days before starting to use dissolved oxygen, but, on the average, by the end of 10 days the activity had increased to about 0.8 that of sewages at pH 5.1 and 7.2. At pH 9.5, the lag period was only 1 to 2 days, although at the end of 10 days activity had developed to only about 0.6 of that of sewages at pH 5.1 and 7.2.

When mold seeds and sewage seeds were mixed, other nutritional factors became operative. In addition to the glucose and glutamic acid of the experiment, the molds received various nutrients from the dilute sewage which was added. Through secretion of exoenzymes, molds may be able to utilize some of the sewage organisms as food, while some of the sewage organisms may be able to prey on the mold seed. On the average, the 10-day B.O.D. of mixed mold seed and sewage seed was lower at pH 2.9, 5.1 and 7.2 than that of sewage seed alone. The same can be said of the L values, which, however, were higher than that of mold seed alone. At pH 9.5 the addition of mold seed to sewage seed sped up the process of utilization of dissolved oxygen to the extent that the mixture used more dissolved oxygen than the sewage seed alone, while the mold seed alone used an insignificant amount of dissolved oxygen.

The ultimate B.O.D. of the glucose and glutamic acid solution in the presence of mold seeds, sewage seeds, or combinations, varied with pH, depending on the inoculum or seed used. The rate at which this demand was

exerted fell within the range of rates to be expected from river water. The demand values could be determined in 101 of the series of B.O.D. tests reported. They could not be determined for the 8 mold seeds at pH 9.5 because insufficient activity occurred. They could not be determined for 8 of the other B.O.D. series because values for entering moments method curves based on either method of calculation described previously were either too high or too low. Other methods of applying k and L values may be applied, but were not used in these studies because insufficient replicate experiments were completed for critical evaluation of the values.

Summary

When compared with the results of Butterfield and Purdy (1931), it appears that the oxygen-depleting capacities of fungi used in this work equal or exceed those of B. aerogenes and other bacteria in pure culture at pH 7.2. Fungus activity in pure culture increased as the reaction of the medium became more acid and decreased as it became more basic. When used in mixed culture with 24- or 48-hour settled sewage, fungi usually gave lower B.O.D. values than the sewage seed alone, except that at pH 9.5 higher values were given.

By themselves, mold seeds produced in most cases a lower B.O.D. than did sewage seeds alone or in mixtures of mold seed and sewage seed. Results indicate that with only molds significant reductions in dissolved oxygen can be achieved in the first four days of incubation at pH 2.9, while the greatest reduction with sewage seeds alone occurs at pH 5.1 and 7.2. This can be attributed to the fact that molds in general are more tolerant of low pH levels than are most other organisms found in sewage.

The strains of fungi tested are able to complete successfully with

other microorganisms for organic materials in solution and for oxygen available only in the dissolved state.

E. Removal of ABS from Solutions by a Common Fungus of Sewage *

Among the more persistent chemicals commonly encountered in water and waste is the surface active agent commonly referred to as alkyl benzene sulfonate (ABS). Thus when ABS was removed rapidly, or was present in much lower concentrations, in the effluent than the influent in more than one experimental sewage treatment system at SEC involving the use of a lysimeter, sandy soil and effluent samples were processed for microbiological populations and absorption phenomena. Sand was obtained from the California sand dunes in the vicinity of the Hyperion sewage treatment plant (Lavery, Stone and Meyerson, 1961). In this experiment, samples of sand from the top and from the bottom of a 6 inch sand bed and samples of the effluent were plated with neopeptone-dextrose-rose bengal-Aureomycin agar for development of fungal populations.

A number of kinds of filamentous fungi and a few yeasts were found to be present. On the assumption that abundance (growth) might be an index of the ability of a fungus to use the ABS in addition to other substances present, or at least to tolerate the ABS, eight of the more common molds that were isolated were incubated into a broth containing 10 p.p.m. ABS as a carbon source and neopeptone as a nitrogen source (although it could also yield carbon). Good growth of all cultures was obtained. Because of growth that might have developed from carbon assimilated from the neopeptone, the supernatant was not tested for ABS.

* Reproduced with permission from *Mycopathologia et Mycologia Applicata* 19:287-295, 1963.

Technique

Because it was among the more common species isolated from the sand bed materials, and because it had grown well in shaken cultures with ABS-neopeptone broth, Phialophora jeanselmei was chosen for additional testing. Ten cultures, including the one isolated from the experimental sand beds, were used in the first experiment. Following an initial test with these 10 strains, five were chosen for additional studies on the basis of their abilities to reduce the amount of ABS in the nutrient solution. Except where noted, growth in these experiments was carried out under room conditions of temperature and light, on a rotary shaker at about 150 oscillations per minute. Composition of the nutrient solution varied only in the amounts of glucose used, unless otherwise noted. It was:

*ABS to give a conc. of 10 p.p.m.	16.4 mg
(NH ₄) ₂ SO ₄	2.0 g
KH ₂ PO ₄	1.0 g
Microelements**	2.0 ml
Distilled water	1000.0 ml

*ABS supplied by the Association of American soap and Glycerin Producers. It consisted of 61.0% ABS, 38.1% Na₂SO₄, 4.7% water, and 2.5% impurities; the ABS was manufactured from tetraporpylene and was considered a "hard" ABS.

** Solution suggested by Lilly and Barnett (1951).

To compare the action of each culture on three media, solutions with no glucose, 1% glucose, and 10% glucose were used.

Throughout the series of experiments, continuous cultures were prepared by inoculating succeeding experiments with growth from the preceding experiment. Because of other scheduling, inoculations of new media and harvesting of old cultures were effected on Wednesday of each week, with two exceptions. In every instance, 1 ml of inoculum was removed from the week-old flask as inoculum for the new medium. Strain 1 in medium 1 was used to inoculate the first flask of fresh medium 1, and so on for the 15 cultures maintained. In this way, fully acclimatized inoculum was available throughout the experiments.

In order to test for ABS in the residual medium or in extracts from the mycelium, the methylene blue test described in Standard Methods of Water Analysis, as modified by Kroner, was used. This technique is:

Place 100 ml of a sample in a separatory funnel. Add 1.0 ml of 5N sulphuric acid and add 5.0 ml of methylene blue solution to each sample and the standards. Mix well. Add 10 ml chloroform to each funnel, invert, and shake once a second for 25 seconds. Allow the chloroform layer to separate. Draw off the chloroform layer and filter through a plug of absorbent cotton into a 50 ml. volumetric flask. Repeat the extraction twice with 10-ml portions of chloroform, collecting the extracts in the same flask. Rinse the cotton plugs with chloroform into the 50 ml volumetric flask, make up to mark with chloroform, mix well, and let stand for 5 to 10 minutes. Read the optical density of each sample and standards against chloroform as a

reference blank at 650 mu in a suitable photometer or respirometer and calculate mg/l of ABS.

Media were prepared in sufficient quantity so that 6 flasks, each containing 50 ml of solution, could be inoculated simultaneously. In the earlier phase of this series solutions were autoclaved, later they were filter sterilized using a membrane filter and sterilized glassware. Controls and standards were also prepared. Fresh solutions were prepared each week.

In one experiment, fresh solutions were inoculated with 1 ml of growth from previous experiments with all 18 of the tested cultures. These flasks were placed in a 37°C incubator, under still conditions, for a 2-week period. No apparent growth could be observed, even in 10% glucose, and incubation under ambient conditions of temperature and light was considered justified.

Results

Shaken Culture

Table 99 presents the results of 17 weekly serial incubation tests. In the first six tests the molds were inoculated into autoclaved media. Lines 7 and 8 of Table 99 represent results from paired tests with autoclaved and filter-sterilized media. Because results in line 8 represent a decrease in ABS over those in line 7, subsequent tests were made with filter-sterilized media, since it appeared that coldsterilized ABS is more subject to attack than that sterilized under heat.

Table 99. AMS Remaining in Solution after 7-day Growth Periods, in ppm.
(Number incubation at room conditions of light and temperature.)

Test Date		Culture Number														
		1	2	3	4	5	6	7	8	9	10					
No	12-19/12-26	Medium														
		10 ppm AMS, 15 glucose														
1	12-19/12-26	8.1	10.0	9.5	9.5	8.7	9.8	5.7	2.9	3.3	3.8	10.5	5.9	6.5	1.7	1.8
2	12-26/1-4	7.3	6.3	7.3	7.4	6.9	1.8	1.3	1.5	2.3	1.9	1.3	2.0	1.0	1.6	1.2
3	1-4/1-11	8.2	8.5	8.8	8.0	8.2	4.5	5.7	3.7	6.6	4.1	5.1	4.5	4.7	5.8	3.1
4	1-11/1-17	8.0	8.3	7.5	8.2	8.2	6.4	5.9	6.7	6.7	6.3	2.4	2.2	3.0	4.6	2.4
5	1-17/1-24	8.1	7.4	7.6	7.6	7.4	4.4	5.0	4.3	6.2	4.5	2.5	2.7	2.4	3.5	2.7
6	1-24/1-31	7.7	7.6	7.5	7.6	7.7	4.6	4.9	4.9	5.1	6.0	2.5	2.7	2.4	3.5	2.7
Sterilization																
7	1-31/2-7 best	7.3	7.9	7.5	7.7	5.3	4.8	6.2	7.5	6.9	5.9	3.8	4.5	5.0	5.1	4.1
8	2-7/2-12 cold	5.2	6.5	5.8	6.9	6.3	6.4	4.5	4.9	4.8	4.6	2.3	2.9	3.4	2.2	2.3
9	2-12/2-21	9.2	8.8	9.5	9.1	8.1	6.6	6.4	6.6	6.4	5.3	2.4	2.8	2.2	0.8	0.9
10	2-21/2-28	8.0	9.1	9.1	9.0	9.3	5.2	3.0	2.6	3.2	2.4	2.0	0.8	0.7	0.2	0.2
11	2-28/3-7	4.8	5.5	3.9	5.5	3.9	1.3	1.2	1.2	0.6	0.7	0.8	0.4	0.7	0.4	0.2
12	3-7/3-14	4.5	4.3	4.9	6.5	5.2	1.6	1.2	2.1	0.8	0.9	0.2	0.2	0.3	0.3	0.2
13	3-14/3-21	4.1	4.8	5.7	4.8	6.7	1.1	3.8	1.3	0.3	1.3	0.6	0.4	0.2	0.3	0.6
14	3-21/3-28	4.0	4.7	4.4	4.4	4.9	1.1	0.8	0.6	0.4	0.4	0.7	0.7	0.2	0.2	0.7
15	3-28/4-4	3.4	2.2	2.2	1.7	4.0	2.4	2.0	2.5	1.8	1.3	0.7	0.7	0.2	0.3	0.7
16	4-4/4-13	6.2	5.7	7.1	6.5	4.9	2.2	2.0	10.2	9.5	7.9	2.4	2.8	2.2	0.8	0.9
17	4-13/4-16	8.1	8.1	8.2	8.2	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1
1	4-13/4-17	7.6	7.6	7.6	7.6	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9
4-13/4-18		7.8	7.8	7.8	7.8	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1
4-13/4-19		7.1	7.1	7.1	7.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1
4-13/4-20		7.1	7.1	7.1	7.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1
Av. AMS remaining																
12-19/1-13	6.6	6.8	6.8	6.9	6.7	6.7	4.5	4.0	3.8	3.8	3.5	2.4	2.1	3.0	1.9	1.4

Cultures inoculated on Feb. 7 were harvested on the 5th day of incubation and on successive days thereafter. No progressive reduction in ABS occurred; rather after 4 or 5 days of incubation there was no change in removal of ABS from solutions. As each culture was harvested in this experiment, a new medium was inoculated with 1 ml of spore suspension from the old and these media were harvested 5 to 9 days after inoculation. Results showed no essential difference between these long-term incubation periods. The inoculations of April 11, refrigerated until April 13 and then incubated for 3 to 7 days on the shaker, did not show a progressive decrease in ABS in the filtrate for the first 6 days of the experiment.

Table 101 presents data from the final ABS experiment. The flasks, each with 50 ml of solution were inoculated on April 11 from cultures harvested that date. They were stored in the refrigerator until April 13, when they were placed on the shaker, and then one set was harvested each day from April 16 through April 20. The maximum and minimum dry weights of growth and amount of ABS remaining in the filtrate occurred in the cultures harvested April 19. After the oven-dry weights were obtained the mycelium from each flask was extracted as before. The amounts of ABS in the solvent from these flasks indicated that only barely detectable amounts of ABS were adsorbed on the mycelia.

In Table 102 data from two of the experiments toward the end of the series are presented. Within each level of glucose concentration, there was considerable flexibility of the ability of each culture to utilize, adsorb or remove ABS from solution, and even to produce mycelial or spore growth in each medium. Best results were obtained with cultures in which there were high concentrations of glucose.

Table 100. Loss of AMS from still culture solutions.

Culture Number	Initial AMS PPM	Glucose \$	Oven dry Mycelium Wt. G.	AMS Remaining in Filtrate \$	AMS Extracted from Oven Dry Mycelium PPM \$	Total AMS Accounted for, \$	AMS Not Accounted for, \$
7.1	10	10	1.8658	15.0	4.56	63.5	36.5
7.2	10	10	1.375	15.6	4.46	62.1	37.9
8.1	10	10	0.7000	22.6	3.26	56.6	43.4
8.2	10	10	0.8439	21.0	4.39	66.7	33.3
6	0	10	.7216	0		0	0
6	10	0	.0013	60.9	1.22	82.12	17.9
6	10	1	.1457	37.5	32.62	70.81	20.0
6	10	10	.5713	17.7	57.09	74.79	25.2

Table 101. Loss of AMB from shrimp culture solutions.

Culture Number	AMS PPM	Glucose %	Growth Period													
			4-13/4-16			4-13/4-17			4-13/4-18			4-13/4-19			4-13/4-20	
			Dry Wt.	AMS in	Dry Wt.	AMS in	Dry Wt.	AMS in	Dry Wt.	AMS in	Dry Wt.	AMS in	Dry Wt.	AMS in	Dry Wt.	AMS in
			Growth	Filterate,	Growth	Filterate,	Growth	Filterate,	Growth	Filterate,	Growth	Filterate,	Growth	Filterate,	Growth	Filterate,
			in mg.	PPM	in mg.	PPM	in mg.	PPM	in mg.	PPM	in mg.	PPM	in mg.	PPM	in mg.	PPM
6	10	0	2.0	8.7	2.0	8.2	1.3	7.6	1.1	7.8	2.1	7.1	2.1	7.1	2.1	7.1
6	10	1.0	6.2	10.2	10.2	7.1	8.4	5.9	12.6	5.0	7.9	6.1	7.9	6.1	7.9	6.1
6	10	10.0	18.8	9.5	51.3	7.9	33.9	5.9	47.1	2.1	41.7	4.4	41.7	4.4	41.7	4.4

Table 102. Amounts of growth produced in AMS medium Culture Solutions.

Culture Number	AMS FPN	Glassness %	Growth Period 3-28-62/4-4-62 Total Dry Wt. of Mycelium in mg.	FPN AMS Remaining in Filtrate after 7 Days	Mycelial Extract AMS, FPN
1	10	0	17.8	3.4	1.0
2	10	0	10.6	8.2	3.0
3	10	0	12.3	2.2	0.4
7	10	0	15.5	1.7	0.1
8	10	0	12.0	4.0	0.4
1	10	1	38.6	2.4	1.3
2	10	1	35.9	0.8	1.1
3	10	1	40.5	0.6	0.3
7	10	1	68.9	0.4	0.3
8	10	1	73.7	0.4	0.1
1	10	10	95.3	0.7	0.2
2	10	10	106.4	0.7	0.2
3	10	10	109.5	0.4	1.9
7	10	10	137.6	0.2	2.0
8	10	10	108.2	0.6	1.6
Growth Period 4-4-62/4-13-62					
1	10	0	2.2	6.2	
2	10	0	2.4	5.7	
3	10	0	0.7	7.1	
7	10	0	1.5	6.5	
8	10	0	2.0	4.8	
1	10	1	32.2	2.2	
2	10	1	18.6	2.0	
3	10	1	21.1	2.5	
7	10	1	23.4	1.8	
8	10	1	32.7	1.3	
1	10	10	93.2	0.7	
2	10	10	122.5	0.7	
3	10	10	114.7	0.2	
7	10	10	108.7	0.3	
8	10	10	116.9	0.7	

Still Culture

Small amounts of ABS remained in the medium after several weeks of serially inoculated incubations (Table 99). Two types of tests were made to determine whether the differences were due to the adsorption of ABS on the mycelium or spores of the fungus, rather than to assimilation by the fungus of ABS as a source of carbon; that is, to answer the question whether we are concerned with adsorption, metabolism, or both. In the first test the fungus was grown in a liter of medium in large flat-bottomed flasks. In the second, the fungus was grown in 50 ml of medium in 250 ml flasks on the shaker. In a Soxhlet apparatus, oven dried mycelium from medium that had been filtered through a membrane filter, was extracted for 7 hours with benzene-methanol-0.4N ammonium hydroxide solvent. The extractant was tested for ABS. It was assumed that ABS was the only extractable material present in the tested solution (Tables 100 and 101). No ABS-like substances were produced by the mycelium either free or in the solution, or adsorbed on the mycelium, in the second of two tests in which the mold was in still culture in the large flasks.

Table 100 gives the results of two experiments with cultures of Phialophora jeanselmei in 2.5 liter flat-bottomed flasks. Each flask contained 1 liter of medium. In the first test, flasks were inoculated in pairs with strains 7 and 8 of this series. All flasks contained 10 ppm ABS and 10% glucose. In the second test, culture 6 of this series was used. In the first flask of this series no ABS was included, 10% glucose being the sole carbon source. In the other three flasks, 10 ppm ABS were present; no glucose was added to one flask, and 1 and 10% respectively were added to the other two. In the first test about the

same amounts of ABS were removed from solution either by metabolic processes or by adsorption, although strain No. 8 grew more slowly than No. 7. In the second test no ABS-like substance was produced by the fungus in the absence of ABS; as the sugar content of the medium increased the ability of the fungus to remove ABS from solution appeared to increase, even in still culture. ABS inhibited the growth of the fungus to some extent, since less tissue was present at the end of 2 weeks in the presence of 10 ppm ABS than in the absence of this substance in the 10% glucose solution.

Discussion

Throughout these experiments, little uniformity was noted in the pattern of ABS removal from solution, either by assimilation or adsorption, on a week-to-week basis or on a strain-to-strain basis. Each fungal strain showed weekly variations on each medium. There was a tendency, however, toward a more or less uniform pattern of ABS usage as the culture was grown for a longer time. This is shown in the final line of Table 99 in which are listed the averages of ABS remaining after 17 weeks of culture.

Some theoretical considerations may be of interest for those wishing to develop a more efficient pure culture. In preparing inoculum for this experiment, a needleful of fungus cells, including both mycelium and spores, was transferred for each culture. While it is definitely established that each of the strains represents the same species, this does not mean that each cell among the thousands that were inoculated into each flask from the parent slant had the same genetic constitution at either the morphological or physiological level. The strain differences between daughter cells are usually less pronounced morphologically than

physiologically, since the general aspect of the progeny cultures is similar. Morphological differences appear in the ability of a strain to produce conidiophores, spores, wall thickenings, yeast-like cells and other aspects of growth patterns. Physiologically, strains may differ in ability to metabolize one or another substrate, rate of use of a nutrient, ability to produce a pigment, vitamin, or other characteristic. When many cells, each of several groups with different characteristics, are brought together from a random population, less consistency of activity will be found than if all the cells had the same initial potential.

Since it may be presumed that Phialophora jeanselmei, an imperfect fungus, is a heterocaryotic fungus, then more than one strain-types may be intermingled in one isolate, and each isolate or strain will have different potentialities. So long as no attempt is made to select a strain with maximum potentialities in one activity or another, irregular results, such as those presented here, are bound to result.

One fact of interest in these experiments is the observation that, as glucose concentration increases, ABS loss in the medium increases. Of course, in normal treatment processes large concentrations of sugar would not be present, or if so their effects would tend to clog filters or bulk sludges by the rapidity of growth of other organisms. Phialophora jeanselmei is a relatively slow-growing organism. It would tend to become crowded out in a mixed population in which organisms with a better competitive advantage existed, or if it remained its influence might be less strongly exerted.

In still culture on the laboratory bench, a definite absorption of ABS on the mycelium and spores of the fungus tissue can be measured (Table 100); this is not observable microscopically as are adsorbed

lignin-containing materials. On the shaker, such adsorption is low or negligible. This could indicate that with continual agitation the mycelium in the flask on the shaker is washed with nutrient in which the oxygen supply is continually renewed, whereas in still culture constant micro-environmental changes do not exist. Such changes may be associated with the ability of the fungus to break down the ABS in its environment.

Because of the preliminary nature of these experiments in which only one fungus in still cultures and in cultures shaken at 150 oscillations per minute on the rotary shaker were examined, it is not practical at this time to indicate theoretical levels of adsorption of ABS on fungal mycelium in either type of experiment. One-third to almost one-half of the ABS in these experiments was adsorbed on the mycelium in still culture when the fungus was grown with glucose in quantities of nutrient as large as 1 liter. When 50-ml units of nutrient solution were inoculated and grown on the shaker much less growth occurred, and when this growth was extracted only barely detectable amounts of ABS could be recovered from the growth although more than half the initial ABS remained in the nutrient solution.

These results indicate the possibility that, in a well aerated system, in which there is a good supply of glucose, certain organisms potentially present in the populations of a sewage treatment system are capable of removing ABS from solution through metabolic processes.

Conclusions

Phialophora jeanselmei is very common in sewage treatment systems, and can remove ABS from solutions even in the absence of glucose. Because glucose is present in sewage treatment systems either as a pollutant or as

a byproduct of the metabolism of other organisms, it appears that, to some extent, ABS may be removed by this organism. Additional work is needed to determine whether this organism can be made a nucleus of an artificial population deliberately established to act upon some special types of wastes; whether a mononucleate-monospore culture capable of more rapid and more complete removal of ABS can be developed; and what pathways of metabolism and decomposition of ABS might be involved with the idea of breaking into the chains of reaction with other organisms.

In the presence of added glucose, Phialophora jeanselmci was found to be capable of removing ABS from shaken and still culture solutions containing an ABS concentration of 10 ppm. More adsorption on the mycelium took place in still culture than in aerated culture. In shaken culture a greater loss of ABS from solution resulted; this may be due to metabolic processes since the ABS was neither found in solution nor adsorbed on the mycelium. While wild cultures are capable of a demonstrable amount of activity, culture efficiency could be improved if selected nononucleate-monospore cultures were developed.

F. The Growth of Yeasts at 37°C *

One factor characterizing several filamentous fungi that cause systemic disease in man is the ability to grow in a yeast-like phase on certain media and under cultural conditions in pure culture. In man, and sometimes in other warm-blooded animals, these fungi grow only in the yeast-like phase. At room temperature, in pure culture, these fungi grow only as filamentous molds. Some molds, including species of Mucor, can grow in a yeast-like phase under special conditions of temperature,

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medium composition, oxygen tension, or carbon dioxide content of the culture atmosphere. Those fungi referred to as yeasts, however, grow in a yeast-like phase at all temperatures at which they are capable of growing, the only morphological change in strains of some species being the loss of production of pseudomycelium.

Many yeasts are capable of growing at 37°C or at higher temperatures. In some types of work these may be considered to be able to grow in man, or at least in warm-blooded animals. While these may not be pathogenic for man, they, or some of them, may be considered to be part of the saprobic biota of the respiratory tract, the alimentary canal, and other surfaces or organs. When the delicate balances of the interactions among various members of this population are upset by pathological conditions or by the techniques used in the treatment of such conditions, some of these organisms may also become involved in the pathogenic condition.

In studies with fungi isolated from sewage and sewage-polluted waters, such as are found in sewage treatment systems and streams carrying raw sewage or sewage treatment plant effluents that have not been properly chlorinated, it might be of interest to know which species are capable of living at elevated temperatures, since among these species may be ones which had recently been intimately associated with warm-blooded animals, including man. Such species may then be useful as possible indicators of pollution of fecal origin.

At the present time more than 50 types of tests are used in developing information upon which to base a description of, or an identification

of, a strain or an isolate of yeast. These include morphological tests and observations, as well as information concerning physiological tests such as ability of the strain to use carbon- and nitrogen-containing compounds, vitamins, and other substances. An additional test that may become useful as more is learned of its reactions in more and more strains of a species or in more and more species of yeasts is the ability of a strain to grow at various temperatures. At present, at least two types of temperature incubation tests are used: temperatures up to 32°C, represented at times by room temperature, and temperatures up to 42°C, represented by 37°C. When equipment is available a range of temperatures may be tested simultaneously. We are not concerned here with potentially cryogenic species. The current report is based on growth on agar media in a 37°C incubator.

As recently as 1952, temperature studies were not considered of importance for yeast identification or classification work. The only temperatures cited by Lodder and Kreger-van Rij (1952) for such studies are 17°C and 25°C. Both may have developed from accidental discovery of optimal growth appearances at room temperature and in a storage room that happened to have a temperature lower than that of room temperature. One of the earlier reports of growth temperatures of yeasts is that of Rennerfeldt, who in 1942, found that four species of yeasts commonly isolated from wood chips grew best at 22°C and at 27°C. In strictly yeast literature, the first report of the use of elevated temperatures in the description of a yeast species is that of Van Uden and Do Carmo-Sousa (1957), who described Candida slooffii from the intestinal tract of a horse. In 1958, Van Uden and Farinha elevated C. norvegensis from varietal rank under C. zeylanoides to

specific rank on the basis of vitamin requirements and the ability to grow at higher temperatures than the former parent species. Again in 1959, Van Uden and Do Carmo-Sousa, working with Candida parapsilosis and a related group of strains, recognized two varieties and two additional species, including C. lusitanae, on the basis, at least in part, of vitamin requirements and growth temperature levels.

For the present study, no attempt has been made to determine the range of growth of the yeast strains used over a series of temperatures. Following inoculation of plates they were incubated at 37°C for 3 days. Thus we were trying to determine only the ability of a strain to grow at 37°C.

The strains used were those routinely isolated from samples of sewage, sewage-polluted waters and soils, and materials obtained for study from various types of sewage treatment processes including trickling filter- and activated sludge-type sewage treatment plants, waste stabilization ponds, soils on which spent sulphite liquor had been sprayed, and so forth. A few other strains on hand were included as the test was used in routine identification procedures.

Inoculation of plates used for temperature studies was inserted into the replicator series described earlier. At first, YNB-glucose medium was used for incubation at 37°C, but growth was relatively slow and generally unsatisfactory. Finally, it was found that a better medium for work at higher than room temperatures was:

Glucose	20 g
Peptone	10 g
Yeast autolysate	5 g
Distilled water	1000 ml

This medium may be made up double strength, dispensed at the rate of 42 ml per tube (25 x 150 mm screw-cap tubes may be used), autoclaved, and stored in the refrigerator until needed. When ready to make up plates, add 10 ml of 2X strength nutrient solution and 10 ml of sterile 2X strength 2% water agar, swirl, and allow to harden. To facilitate mixing the agar should be hot.

After the 3-day incubation period the plates may be removed from the incubator and read, comparative estimate values being given for amounts of growth observed. Maximum growth is recorded as +++, good growth as ++, poor growth as +, no growth as zero (0), or -. If considered of interest, trace amounts of growth may be recorded as T. The first two categories are considered as indicative of the ability of the yeast to grow at 37°C, the other categories are not. On this basis the values presented in Table 103 were obtained. If 0, T, or + amounts of growth were recorded, the strain is not included among those for which a percentage of strains gave satisfactory growth at 37°C.

If it is assumed that all strains of one species are able to exhibit all growth responses expected of that species, then more than one species is represented in most of the categories presented in Table 103. If, however, we assume that all strains of a given species need not react positively to all details of a growth pattern established for that species, then we can see that in any species for which sufficient strains have been tested there are strains that do not react according to the pattern established for the species. This is true of many of the less important physiological tests as well as of the 37°C test, and there are some strains of species

Table 103.

Growth of yeast species at 37°C. *

Yeast Species	Number of Strains Tested	No growth or weak growth Number	Growth at 37°C		Percent of Total Growing at 37°C
			Medium or Strong Growth Number		
<u>Candida curvata</u>	35	31	5		13.9
<u>guilliermondii</u>	98	3	95		96.9
<u>humicola</u>	16	11	5		21.3
<u>intermedia</u>	23	18	5		21.7
<u>krusei</u>	366	33	333		91.0
<u>lusitania</u>	2		2		100.0
<u>melinii</u>	2	1	1		50.0
<u>mycoderma</u>	3	2	1		33.3
<u>parapsilosis</u>	192	56	136		70.8
<u>pelliculosa</u>	5		5		100.0
<u>pseudotropicalis</u>	1		1		100.0
<u>pulcherrima</u>	15	4	11		73.3
<u>rauvallii</u>	1		1		100.0
<u>rugosa</u>	9	2	7		77.8
<u>solani</u>	4	1	3		75.0
<u>tropicalis</u>	156	4	152		97.4
<u>utilis</u>	19	5	14		73.7
<u>zeylanoides</u>	1		1		100.0
spp.	30	15	15		50.0
<u>Cryptococcus</u>					
<u>ster</u>	2	2			
<u>albidus</u>	37	35	2		5.4
<u>diffusus</u>	28	27	1		3.6
<u>laurentii</u>	45	38	7		15.6
<u>luteolus</u>	12	11	1		8.3
<u>neoformans</u>	6		6		100.0
<u>Debaryomyces</u>					
<u>hansenii</u>	1		1		100.0
<u>Endomycopsis</u>					
<u>javanensis</u>	5	3	2		40.0
<u>Hansenula</u>					
<u>anomala</u>	15	5	10		67.0
<u>californica</u>	16	13	3		18.8
<u>allipsoidea</u>	2		2		100.0
<u>saturnus</u>	18	15	3		16.7
<u>Kloeckera</u>					
<u>apiculata</u>	11	11			
<u>Pichia</u>					
<u>fermentans</u>	3	3			
<u>membranefaciens</u>	7	4	3		37.5
<u>Rhodotorula</u>					
<u>flava</u>	1	1			
<u>glutinis</u>	191	179	12		6.3
<u>graminis</u>	3	3			
<u>macerans</u>	1	1			
<u>minuta</u>	4	4			
<u>wucilaginosa</u>	350	215	135		38.6
<u>pennaeus</u>	2	2			
<u>pilimanis</u>	2	2			
<u>texensis</u>	13	12	1		7.7
<u>Saccharomyces</u>					
<u>sp</u>	2	1	1		50.0
<u>carlsbergensis</u>	1		1		100.0
<u>cerevisiae</u>	129	12	117		90.7
<u>drasophilus</u>	2		2		100.0
<u>montanus</u>	2		2		100.0
<u>Schwanniomyces</u>					
<u>alluvius</u>	2	1	1		50.0
<u>Sporobolomyces</u>					
<u>salmonicolor</u>	1	1			
<u>Smut-like</u>	56	14	42		75.0

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<u>Torulopsis</u> <u>seria</u>	4	1	3	75.0
<u>candida</u>	45	9	36	80.0
<u>colliculosa</u>	1		1	100.0
? <u>dattila</u>	2		2	100.0
? <u>ernobii</u>	6	5	1	16.7
<u>fasata</u>	115	59	56	48.7
<u>glabrata</u>	53	13	40	75.5
<u>holwii</u>	4	3	1	25.0
<u>inconspicua</u>	43	5	38	88.4
<u>molischiana</u>	2	2		
<u>stellata</u>	2	2		
<u>vansylui</u>	9	9		
<u>versatilis</u>	1	1		
spp.	19	7	12	63.2
<u>Trichosporon</u> <u>behrendii</u>	1		1	100.0
<u>capitatum</u>	3		3	100.0
<u>cutaneum</u>	57	41	16	28.1
<u>fermentans</u>	6	2	4	67.0
<u>margaritiferum</u>	3	1	2	67.0
<u>pullulans</u>	9	8	1	11.1
<u>sericeum</u>	2		2	100.0
spp.	7	3	4	57.1

* Results refer only to cultures listed. Category assignment is tentative in species preceded by a question mark, as well as certain other species.

** Includes Mycoderma lambica.

of Candida that produce only Torulopsis- and even Cryptococcus- like growth.

How many strains of a species need be tested before we are sure that a sufficient number have been tested? The answer to this question is not easy to determine. Certainly it is more than one. Moreover, by the time we have tested 366 we may have tested more than enough. A check of Table / ¹⁰³ shows that more than 2,300 strains of 70 species of yeasts were tested for growth at 37°C. Of these, all strains tested of 15 species grew well, all strains tested of 13 species grew poorly or not at all. None of these species was represented by more than 10 strains each. When we come to species represented by 10 or more strains, none of the 42 species tested produced all good growth or no growth at all. The test of sufficiency will, however, depend in part on the urgency of the need for an answer, in part on the number of strains available for testing, and in part on the performance record of the strains of a species that have been tested. Since few species on the list in Table 103 give a consistent pattern of behaviour at even the level of 10, this may be a convenient number to use. On this basis it can be said that on this single test most species will not react uniformly.

Since in many types of isolation where a control sample of unpolluted material is used to compare a polluted with an unpolluted habitat, species usually thought to be normally associated with man are found, it is suggested that within one species there may be two basic groups of strains, one capable of adjusting to growth conditions at elevated temperatures, the other not. Both types may occur simultaneously in the same sample,

or each type may be restricted to a special habitat. The strain capable of growing at the higher temperature may be suggestive of the occurrence of human or of warm-blooded animal pollution at the point from which it was isolated.

G. Growth and Metabolism of Fungi in an Atmosphere of Nitrogen *

Since fungi, including filamentous species as well as yeasts, are relatively abundant in digesting sludges and other areas in sewage treatment where oxygen supplies are at a minimum or theoretically absent, it was of interest to discover whether these organisms are capable of metabolizing some of the organic components of sewage and industrial wastes under anaerobic or microaerophilic conditions, or whether they are present in a quiescent state as a result of sedimentation processes.

In routine isolations of fungi from various materials in sewage treatment plants, described above, it was found that a considerable number of yeast and mold colonies could be recovered on pour plates from the bottom of sludge digesters, as well as from other habitats considered to be anaerobic. The basis for the assumption of lack of oxygen in the bottom of the digester was the negative result of chemical analysis for free oxygen and the fact that in such complex digester material are large numbers of organic components which immediately utilize free available molecules of O_2 for oxidative reactions. The continuing recovery of fungus spores and cells from digesting sewage and other microaerophilic habitats led us to pursue this point to discover whether or not these fungi could be active under conditions of complete anaerobiosis. It was thought that the knowledge of specific requirements for oxygen and tolerance to high N_2 levels, might be useful in the problem of more

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advantageous utilization of the metabolic capacities of fungi during waste and sewage treatment.

Materials and Methods

The pure cultures of fungi used in this study were selected at random from representative species encountered in: (1) sewage and waste treatment processes with low oxygen tension; (2) acid mine drainage water and sediment; (3) sediments from creek bottoms along the settling basins near a strip mining area; and (4) litter and soil samples from forest areas. Other cultures were isolates obtained from sewage incubated in anaerobic jars on nutrient agar plates in other studies at the Robert A. Taft Sanitary Engineering Center. These cultures were identified as: Aspergillus fumigatus, A. niger, Aureobasidium pullulans, Candida parapsilosis, Fusarium oxysporum, F. solani, Geotrichum candidum, Mucor hiemalis, Penicillium brevi-compactum, P. cyclopium, Phialophora jeanselmei, Phoma herbarum, Rhodotorula mucilaginosa and Trichoderma viride. The list of mold and yeast species included in the study, together with the sources of each of these cultures, are given in Table 104.

The anaerobic equipment consisted of Case "anaero-jars" equipped with aluminum tops, containing vacuum and positive pressure gauges, valve controls, outlet for evacuation, and gas inlets. In the preliminary experiments use was also made of an anaerobic incubator provided with vacuum gauge, valve controls, temperature controls, and cooling accessories. Both the Case anaero-jars and the vacuum incubator were outfitted with a heavy duty vacuum pump. A mercury column and a vacuum gauge were attached to the evacuation lines to determine the completeness of evacuation and the level of gas being added to provide the atmosphere for the growth of the cultures to be tested.

Table 104. List of Fungus Species Included in the Study and their Source of Isolation.

Fungus	Culture Designation	Date of Isolation	Source
<u>Phialophora jeanselmei</u> (Langeron) Samson	A-294 (ATCC 16637)	4-3-52	Sewage treatment plant, Dayton, Ohio Sludge drying bed.
<u>Aspergillus fumigatus</u> Fresenius	B-40-1 (ATCC 16619)	-64	Isolated from sewage in anaerobic jars in Dr. G. Berg's laboratory. On Petri plates with nutrient agar.
<u>Geotrichum candidum</u> Link ex Persoon	C - D8-5 3067 (ATCC 16635)	2-11-58	Digested sludge, Sewage treatment plant, Dayton, Ohio
<u>Aureobasidium pullulans</u> (de Bary) Arnaud	D - T-2-3-5-M-7 (ATCC 16631)	-63	Sludge digestion study. From unsterilized fish meal slurry on neopeptone-dextrose-rose bengal-Aureomycin agar.
<u>Trichoderma viride</u> Link ex Fries	E - AM-5-35 (ATCC 16640)	6-9-64	Acid mine drainage water and sediment at edge of pond in strip mine, Grassy Run drainage, Coalton Dump, Elkins Area, West Virginia.
<u>Aspergillus niger</u> van Tieghem	F - AM-5-33 (ATCC 16620)	6-9-64	Acid mine drainage water from pond in strip mine at Coalton Dump. Grassy Run drainage. Elkins area, West Virginia.
<u>Mucor hiemalis</u> Wehmer	G - T-4-2-M-1 (ATCC 16636)	-63	Sludge digestion study, Draw-off of digested secondary sludge. Isolated on neopeptone-dextrose-rose bengal-Aureomycin agar.
<u>Phoma herbarum</u> Westendorp	H - T-4-2-M-1 (ATCC 16638)	-63	(Same as above)
Mixture of 2 species: <u>Penicillium cyclopium</u> Westling <u>Penicillium brevi-compactum</u> Marck	I - 40-4	-64	Isolated from sewage in anaerobic jars in Dr. G. Berg's Laboratory, on Petri dishes with nutrient agar.
<u>Rhodotorula mucilaginosa</u> (Jorganson) Harrison	J - T-4-6-Y14-1	-63	Sludge digestion study. Draw-off of digested secondary sludge. 6th draw. Isolated on YNB-1% dextrose; 1:10,000 dilution.

Candida parapsilosis (Ashford)
Langeron and Talice

Fusarium solani (Martius) Appel
and Vollenweber

Fusarium oxysporum Schlechtendahl

K - T-4-2-M-4-1
(ATCC 16632)

L - B4-8-M-1
(ATCC 16634)

M - AMT-14-15
(ATCC 16633)

-63

4-20-64

11-6-64

Sludge digestion study. Draw-off of
digested secondary sludge. Isolated
on neoptone-dextrose-rose bengal-
Aureococcal agar.

Litter in undisturbed chaparral.
Mt. Shasta, Shasta County,
California.

Sediment in creek bottom. Booth Creek
between gab pile and settling basin.
Elkins, West Virginia.

Previously washed and blended cultures of fungi were inoculated into a liquid basal synthetic medium containing various combinations of carbon and nitrogen sources, dispensed in 10 ml amounts in 25 ml conical flasks. The sterile dispensed medium was pre-boiled and cooled down just prior to inoculation. Parallel flasks were used for each combination of carbon and nitrogen source as well as for the basal medium without nitrogen and carbon. The inorganic nitrogen source used was ammonium sulfate, and the organic nitrogen sources were asparagine and glycine. These were selected as optimum nitrogenous compounds from a group of inorganic and organic nitrogen sources which were included in preliminary experiments. Nutrient media were prepared as follows: the carbon source, glucose, was used in the amount of 10 g/l; all nitrogen sources, asparagine, glycine, NH_4NO_3 and $(\text{NH}_4)_2\text{SO}_4$ were used in the amount of 2 g/l; KH_2PO_4 1.0 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and KCl 0.5 g/l; stock solution of trace elements 1 ml/l; thiamine hydrochloride 1 ml of a 10 $\mu\text{g}/100$ ml in 20% ETOH stock solution per liter to give 100 $\mu\text{g}/1$ of medium; and biotin 1 ml of an 0.5 $\mu\text{g}/100$ ml of 20% ETOH stock solution per liter to give 5 $\mu\text{g}/1$ of medium. The stock solution of micronutrients was prepared as follows: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 100 μg ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 880 μg ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 40 μg ; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 15 μg ; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 10 μg ; and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 5 μg , all in 100 ml distilled water. The initial pH value for the media was 3.5, except for media containing glycine where the pH was 4.0.

One series of flasks contained basal synthetic medium with varied carbon and nitrogen source combinations, to which no reductant was added. One-tenth ml of 1% aqueous solution of sodium formaldehyde sulfoxylate

per 10 ml of medium was added to another parallel series of flasks.

The initial pH value was not changed. Flasks containing uninoculated medium, with the reductant Na formaldehyde sulfoxylate and the oxidation-reduction potential indicator methylene blue dye in the leuco form, were incubated in parallel with the inoculated series of flasks to serve as a control for anaerobic (reduced) conditions within the liquid medium in the flasks. In addition a flask with only the reduced form of the methylene blue indicator served as a control for the anaerobic conditions of the atmosphere outside the flasks in the jars and in the incubator.

Prepurified nitrogen gas was introduced to the anaero-jars via needle valve regulators through inlet tubes. In preliminary experiments, as well as throughout a portion of the subsequent studies, the nitrogen gas was passed from the cylinders through a series of three scrubbers in the form of a gas train containing: (1) Zn-Hg amalgam and 0.1 M vanadyl sulfate; (2) 0.1 M NaOH; and (3) H₂O before entering the incubation chambers, to remove all traces of O₂ (Umbreit, Burris and Stauffer, 1957). However, no apparent differences in the reduced state of the atmosphere within the jars and in liquid media, or in the effect on the growth and carbon utilization by the cultures were observed, without the additional purification of cylinder N₂ gas by means of scrubbers.

The anaerobic jars were evacuated and prepurified 100% nitrogen gas was added to give a positive pressure of 10 in. Hg. in each jar. The evacuation was effected four times with the addition of the gas after each evacuation. There was no observable change in color of the

leuco form of the methylene blue indicator, until the jars were opened to remove flasks for harvest, determination of growth and pH and for analysis of the total organic soluble carbon of the media. Each jar was opened only once because it contained only flasks that were to be used for each of the four specified weekly harvests. This prevented the flasks from contacting air throughout the anaerobic incubation period.

Together with the anaerobic incubation, a parallel series of flasks, containing similar media, with and without sodium formaldehyde sulfoxylate, were inoculated and incubated in still culture, aerobically, and similarly, growth, pH, and the total soluble organic carbon determinations were made at weekly intervals for a period of four weeks.

Growth of 13 species of fungi in a defined liquid medium on a rotary shaker in several combinations of carbon and nitrogen sources and pH levels was used to determine the optimum growth promoting conditions to be used in synthetic media for anaerobic studies. The inoculum for anaerobic studies was prepared by using this chemically defined medium and shaking the cultures for 60 hours. The resulting growth was washed twice after centrifuging, the mycelial mass was broken up in sterile triple distilled water in a Waring Blendor, and stored in a refrigerator.

One-tenth ml of the chilled suspension of cells was added to each flask containing 10 ml of the medium, and the flasks were then incubated aerobically and anaerobically at room temperature. At each harvest time, pairs of flasks of each species of fungus and each medium used were

removed from the anaero-jars and contents filtered through a previously weighed membrane filter. Prior to filtration of growth, each membrane filter was washed with 500 ml of deionized, double distilled water to remove the carbonaceous filler material. Oven dry weights were determined and corrected for the weight of the filler material lost by the washing process. This correction factor was based on an average of 20 membrane filters, and was repeated for each batch of membrane filters used. The same procedure was followed with aerobic cultures. In addition to the use of optimal media, the basal medium alone, and each carbon and nitrogen source separately in the basal medium, were used as controls to compare the amount of growth obtained for each species of fungus, aerobically and anaerobically.

At each harvest, a parallel series of flasks was taken from the anaero-jars and incubated aerobically to study the effect of anaerobic preincubation on the growth and metabolic activity of these cultures in air. Growth, pH and total soluble organic carbon determinations were made on these anaerobically preincubated aerobic cultures at the end of 4, 3, 2 and 1-week incubation in air.

The supernatant of the filtered cultures was collected in chemically clean screw-cap tubes. A portion of the liquid was used for pH determinations and the remaining volume was acidified with concentrated H_2SO_4 and analyzed for total soluble organic carbon with the use of a Beckman Model 315 Infrared Carbon Analyzer (1964).

Bartnicki-Garcia and Nickerson (1961, 1962) reported that some fungi require preformed vitamins as growth promoting factors for development under anaerobic conditions. Therefore, it was desirable to

study the growth and metabolic activities of our fungal species anaerobically on media devoid of the vitamins, biotin and thiamine. Accordingly, a parallel series of flasks, containing similar media, with and without sodium formaldehyde sulfoxylate, but without vitamins, were inoculated and incubated anaerobically for a period of 4 weeks. Growth, pH determinations, and analysis of the supernatant for the total soluble organic carbon were made weekly.

Experimental Results

Table 105 compares oven dry weights in mg of cell mass of the 13 species grown both anaerobically and aerobically in media with inorganic or organic nitrogen source, with or without sodium formaldehyde sulfoxylate, and gives a ratio of the anaerobic to aerobic growth for each of these cultures. The species are listed in order of the highest to lowest amount of growth in these media. Based on the ratios of anaerobic to aerobic growth, it can be said that Geotrichum candidum, both Fusarium species, and Mucor hiemalis grew best; Aspergillus niger, A. fumigatus, Aureobasidium pullulans, Phialophora jeanselmei, Penicillium spp., and Trichoderma viride were intermediate; and Phoma herbarum, Rhodotorula mucilaginosa and Candida parapsilosis were lowest in their ability to tolerate and grow in atmospheres of 100% N₂ gas.

Comparisons were made for each of the species, between their growth rates in complete synthetic media, media without the carbon source, media without the nitrogen sources, and in control media, devoid of either the carbon or nitrogen sources, in a 100% N₂ gas atmosphere. The ratios of the growth increase in their atmosphere in synthetic media and in the

Table 105

Comparison of oven-dry weights of cell mass of 13 fungus species grown in still culture, anaerobically in prepurified 100% Nitrogen gas, and aerobically in media with inorganic or organic nitrogen sources and with or without the reductant sodium formaldehyde sulfoxylate.

Fungus	Average weight of cell mass in mg. after four weeks of incubation						Ratio: Anaerobic; Aerobic	
	Anaerobic Growth		Aerobic Growth		Inorganic N			
	Inorganic N	Organic N	Inorganic N	Organic N	Inorganic N	Organic N		
<u>Geotrichum candidum</u>	*20.34	20.15	25.64	43.14	79.3	51.4		
	**19.92	20.85	24.95	41.86	79.8	49.8		
<u>Fusarium oxysporum</u>	17.32	17.45	23.24	30.29	74.5	57.6		
<u>Fusarium solani</u>	17.24	17.38	22.15	20.07	77.8	57.8		
	16.89	16.96	25.60	43.85	66.0	37.8		
<u>Aspergillus niger</u>	16.78	16.85	28.16	39.14	59.6	43.1		
	14.07	14.32	46.12	49.76	30.5	28.8		
<u>Aspergillus fumigatus</u>	13.32	13.90	38.15	46.09	34.9	30.2		
	13.65	13.86	35.72	38.26	38.2	34.4		
<u>Aureobasidium pullulans</u>	12.96	13.08	34.71	38.02	37.3	34.4		
	13.26	13.38	37.84	31.69	35.0	42.2		
<u>Penicillium spp.</u>	13.21	13.28	39.65	34.12	33.3	38.9		
	12.79	12.85	29.26	32.52	43.7	39.5		
<u>Phialophora jeaneelmei</u>	12.62	12.71	29.81	35.47	42.3	35.8		
	12.61	12.76	39.65	26.42	31.8	48.3		
<u>Mucor hiemalis</u>	12.59	12.65	37.05	28.19	34.0	44.9		
	12.36	12.74	21.05	23.12	58.7	55.3		
<u>Phoma barbatum</u>	12.21	12.20	18.91	25.18	64.6	48.5		
	10.73	10.86	28.25	38.14	38.0	28.5		
<u>Trichoderma viride</u>	10.65	10.72	29.27	37.61	36.4	26.7		
	9.47	10.16	19.26	38.05	51.8	35.6		
<u>Rhodotorula marilagoensis</u>	9.83	10.00	17.64	28.05	55.7	24.4		
	6.84	6.95	34.56	28.54	19.9	22.7		
<u>Candida parapsilosis</u>	6.75	6.83	33.07	30.15	20.4	20.2		
	6.02	6.25	25.72	30.89	23.4	19.2		
	5.94	6.03	25.42	31.48	23.4	19.2		

* Media without reductant
** Media with reductant

several control media with and without reductant at the end of each week of incubation were determined for each species and these are presented for four species in Figures 54-57. Graphs prepared for the other nine species are very similar. The addition of sodium formaldehyde sulfoxylate had no significant effect on the growth ratios of each culture in the different media. The organisms giving the highest relative ratios of growth increase in complete synthetic media to growth in the control medium for each week of incubation under 100% N₂ gas were observed to be Geotrichum candidum, Penicillium spp. and Aureobasidium pullulans. The species showing intermediate growth increase ratios are listed in descending order: Fusarium solani, Phialophora jeanselmei, Fusarium oxysporum, Phoma herbarum, Aspergillus fumigatus, Rhodotorula mucilaginosa and Mucor hiemalis, whereas those with the lowest ratios were found to be Trichoderma viride, Aspergillus niger and Candida parapsilosis. The ratios of growth increase in media containing a carbon source only to the growth in control media, were observed to be slightly higher than those in carbohydrate-free media, with either an organic or an inorganic nitrogen source. In all instances, the organic nitrogen source carbohydrate-free media gave relatively higher growth increase ratio values than the inorganic nitrogen carbohydrate-free media. Table 106 gives a comparison of these growth increase ratios to the control medium of the 13 species studied, and relates them to growth values in control minimal basal media. The data represent growth ratios in complete synthetic media with or without the reductant, to growth in control nitrogenless and carbohydrate-free media at the end of 1 and 4 weeks of incubation.

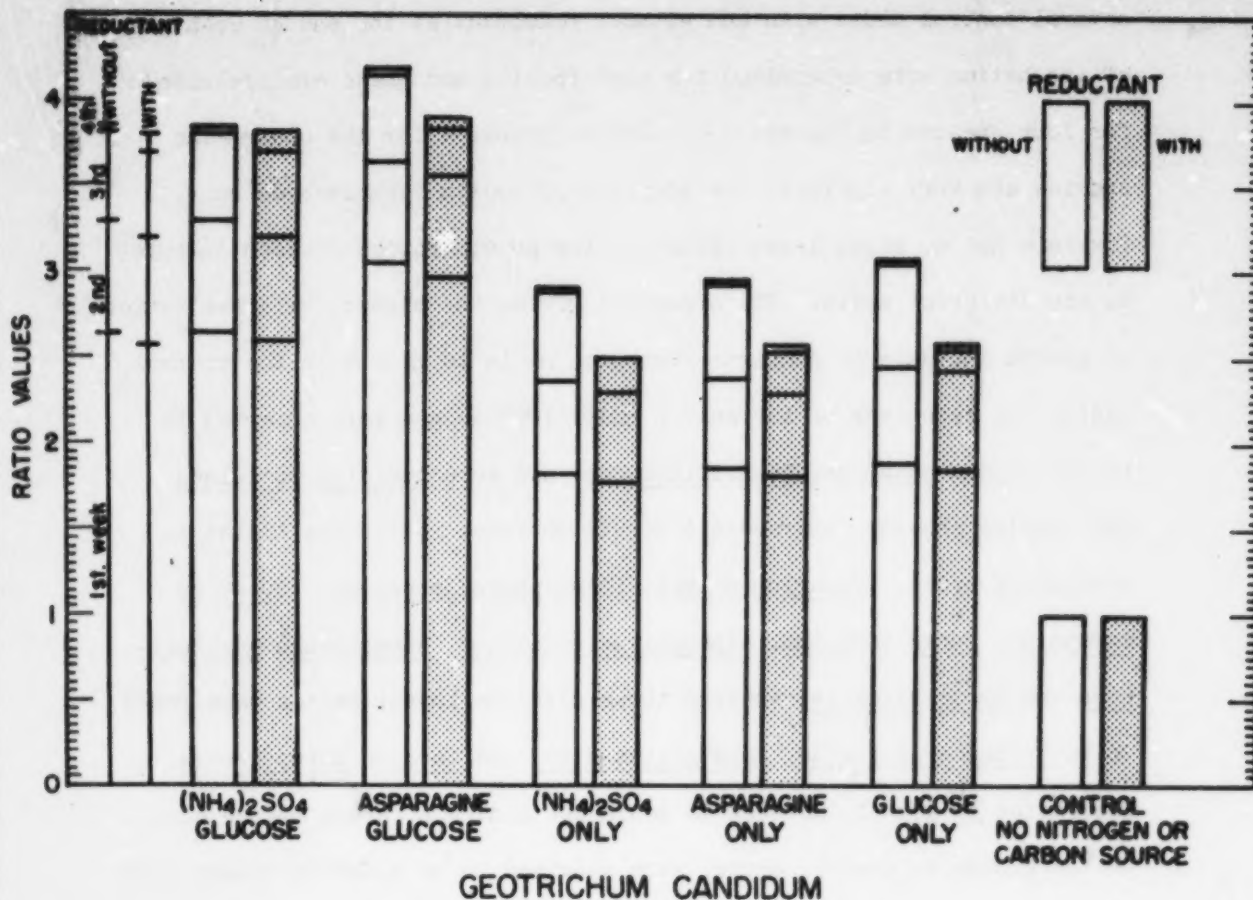
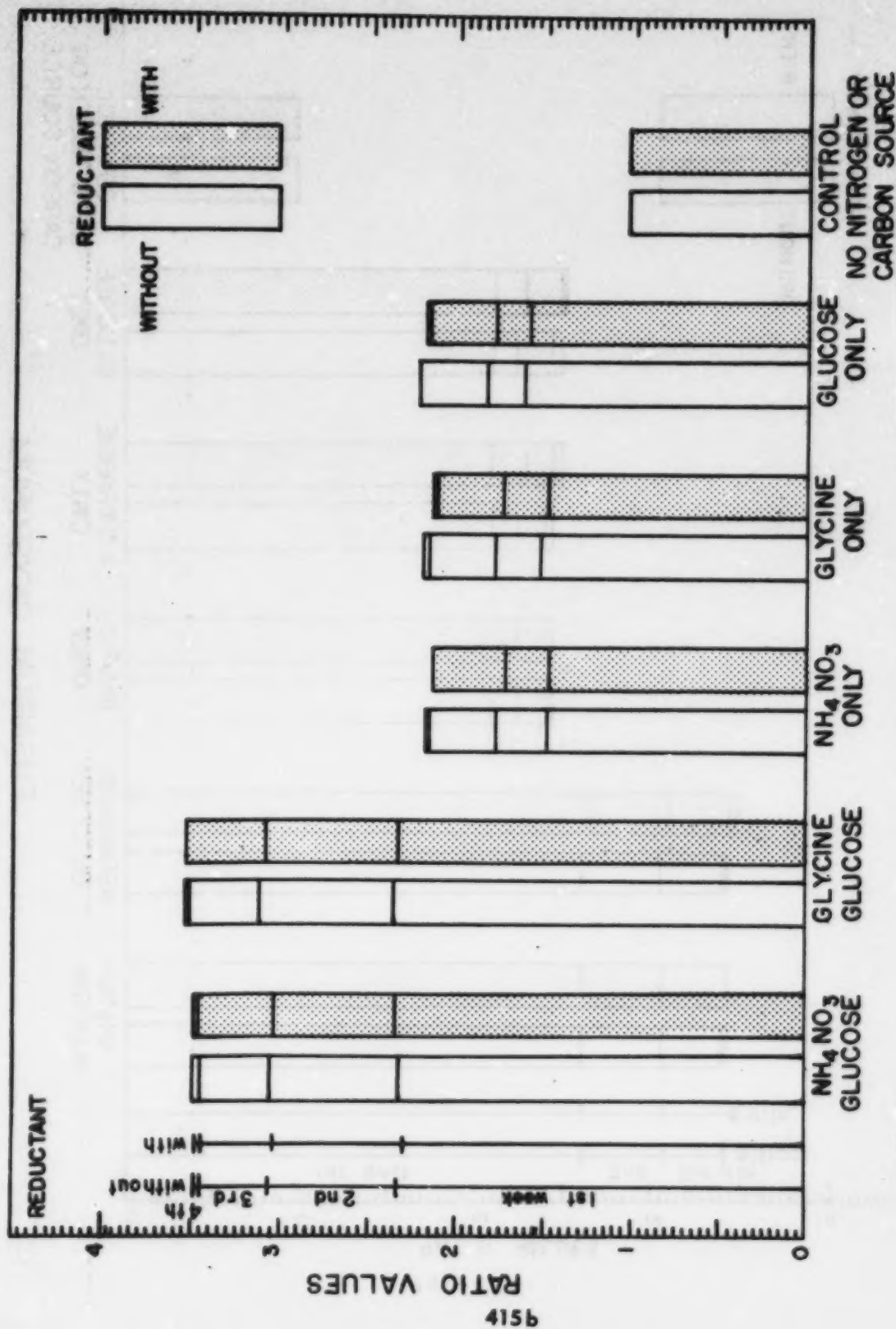


FIGURE 54

Geotrichum candidum. Comparison of the ratios of anaerobic growth increase in complete synthetic medium, carbonless medium, and nitrogenless medium, to growth in the control medium, with and without the reductant sodium formaldehyde sulfoxylate, in an atmosphere of prepurified 100% nitrogen gas at the end of each of 1, 2, 3, and 4 weeks of incubation.

Phialophora jeanselmei. Refer to Fig. 54 legend.



PHIALOPHORA JEANSELMEI

FIGURE 55

Fusarium oxysporum. Refer to Fig. 54 legend.

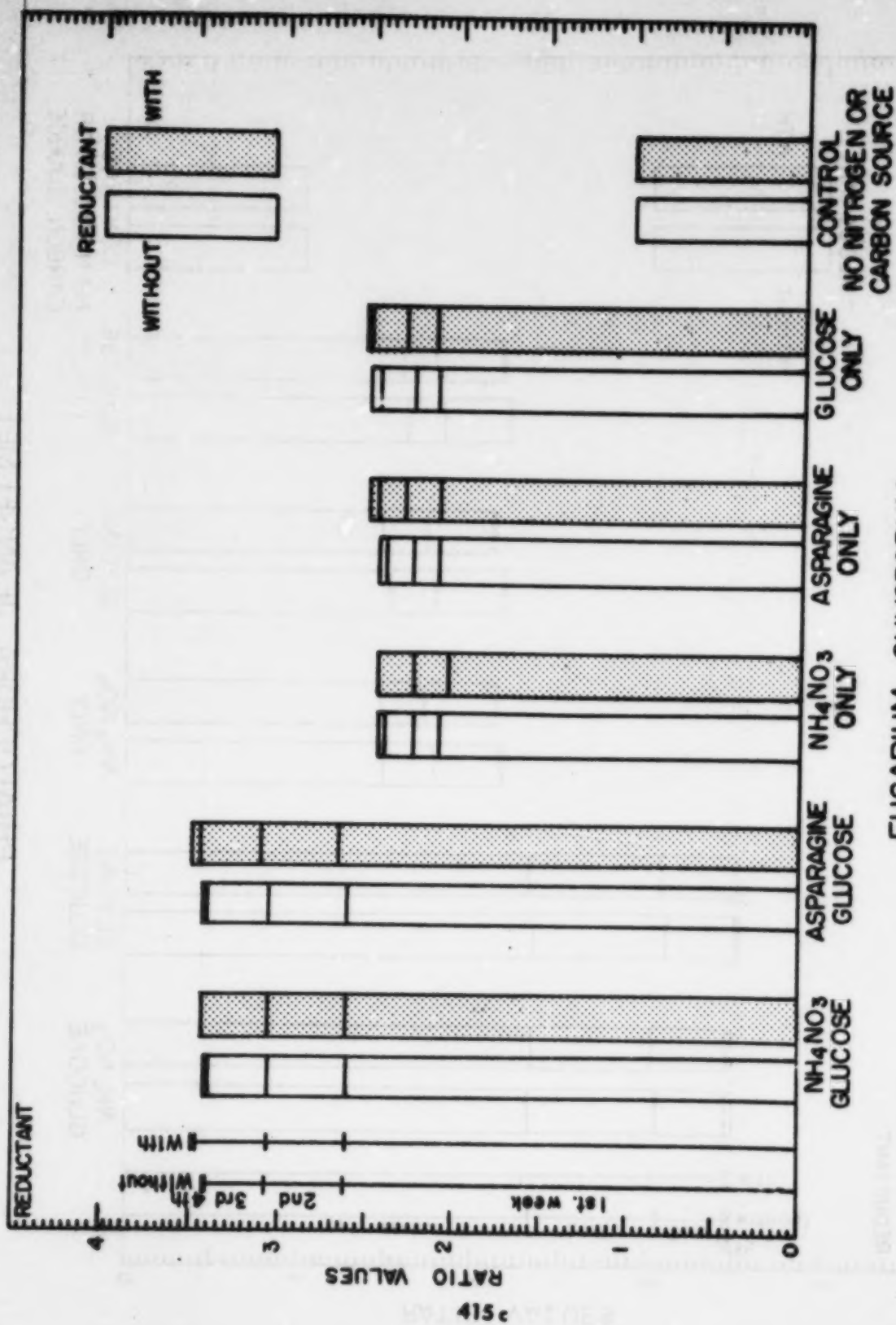


FIGURE 56

Rhodotorula mucilaginosa. Refer to Fig. 54 legend.

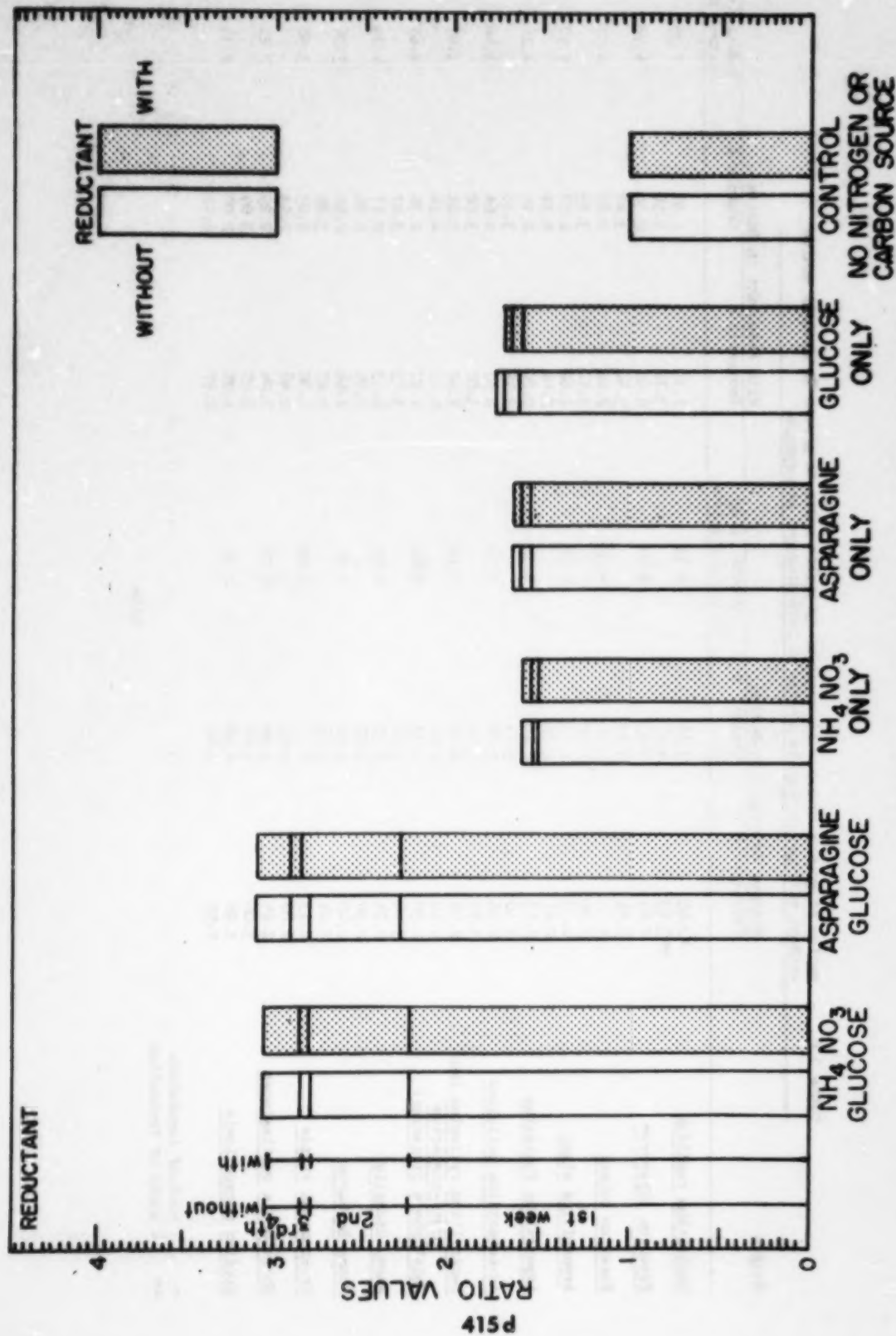


FIGURE 57

Table 106. Ratios of growth increase in complete synthetic media to growth in control media without nitrogen or carbon source in 100% nitrogen atmosphere.

Fungus	Without Reductant in Medium		With Reductant in Medium		Growth in Control Medium
	Inorganic N	Organic N	Inorganic N	Organic N	
<u>Geotrichum candidum</u>	*2.65	3.05	2.59	2.97	5.35
	**3.63	4.17	3.72	3.90	
<u>Fusarium oxysporum</u>	2.65	2.63	2.62	2.66	4.98
	3.41	3.44	3.46	3.49	
<u>Fusarium solani</u>	2.61	2.64	2.62	2.66	4.77
	3.50	3.52	3.52	3.53	
<u>Aspergillus niger</u>	1.73	1.83	1.62	1.72	5.52
	2.61	2.66	2.42	2.52	
<u>Aspergillus fumigatus</u>	2.13	2.18	2.20	2.24	4.16
	3.16	3.21	3.12	3.16	
<u>Ascreosidium pullulans</u>	2.56	2.64	2.57	2.64	3.48
	3.75	3.78	3.80	3.82	
<u>Pendotillium cyclopius and brevi-conspicuum</u>	2.95	2.98	2.99	3.01	3.07
	4.10	4.12	4.11	4.14	
<u>Phialophora jeikei</u>	2.30	2.33	2.27	2.30	3.65
	3.46	3.51	3.45	3.47	
<u>Neurospora crassa</u>	2.24	2.30	2.13	2.17	4.29
	2.90	3.00	2.85	2.86	
<u>Penicillium herveyi</u>	2.46	2.50	2.48	2.50	3.26
	3.24	3.28	3.27	3.29	
<u>Trichoderma viride</u>	2.13	2.37	1.82	2.03	3.96
	2.86	2.91	2.48	2.53	
<u>Rhodotorula mucilaginosa</u>	2.24	2.29	2.26	2.29	2.21
	3.06	3.09	3.05	3.09	
<u>Oospora purpurescens</u>	1.96	2.03	1.95	2.00	2.31
	2.55	2.65	2.57	2.61	

* 1 week of incubation
** 4 weeks of incubation

The ratios of growth increase in complete synthetic media without reductant to control medium, range from 4.17 for Geotrichum candidum to 2.65 for Candida parapsilosis, whereas the ratios in the same media with sodium formaldehyde sulfoxylate give values from 3.90 to 2.61 for the same species.

Values of total soluble organic carbon remaining in media with inorganic or organic nitrogen sources and without the reductant sodium formaldehyde sulfoxylate at the end of 1, 2, 3, and 4 weeks of incubation in an atmosphere of 100% N₂ gas were compared with values of the initial concentration of C/1 in mg in the uninoculated media. Such data also include the values of total soluble organic carbon remaining in media during aerobic growth of 4, 3, 2, and 1 week, following anaerobic preincubation of 1, 2, 3, and 4 weeks respectively. Similar data for all 13 species grown in synthetic media, with an inorganic or an organic nitrogen source and containing the reductant, were also obtained. Results with Geotrichum candidum, Fusarium oxysporum, F. solani, Phoma herbarum, and Trichoderma viride indicate an appreciable loss of total organic soluble carbon in the media after the first week of incubation, followed by a less pronounced loss of carbon at the end of 2 and 3 weeks, and finally a gradual increase of carbon concentration at the end of 4 weeks. In the case of the two species of Aspergillus, Penicillium spp., Mucor hiemalis and Candida parapsilosis, there is an abrupt loss of total soluble carbon in media at the end of 1 week, followed by a more gradual loss throughout 2, 3, and 4 weeks,

without a subsequent increase in concentration at the end of 4 weeks. With the remaining cultures, Aureobasidium pullulans, Phialophora jeanselnei, and Rhodotorula mucilaginosa, there is very little observable loss of carbon in media at the end of 1 week and a subsequent gradual loss after 2 and 3 weeks, followed by a slight increase at the end of 4 weeks. The increase of total soluble carbon in media at the end of 4 weeks of incubation is presumably due to the soluble organic byproducts of the utilization of glucose. The carbon-loss data demonstrating metabolic activity in media with the reductant are not significantly different from carbon data in media without reductant.

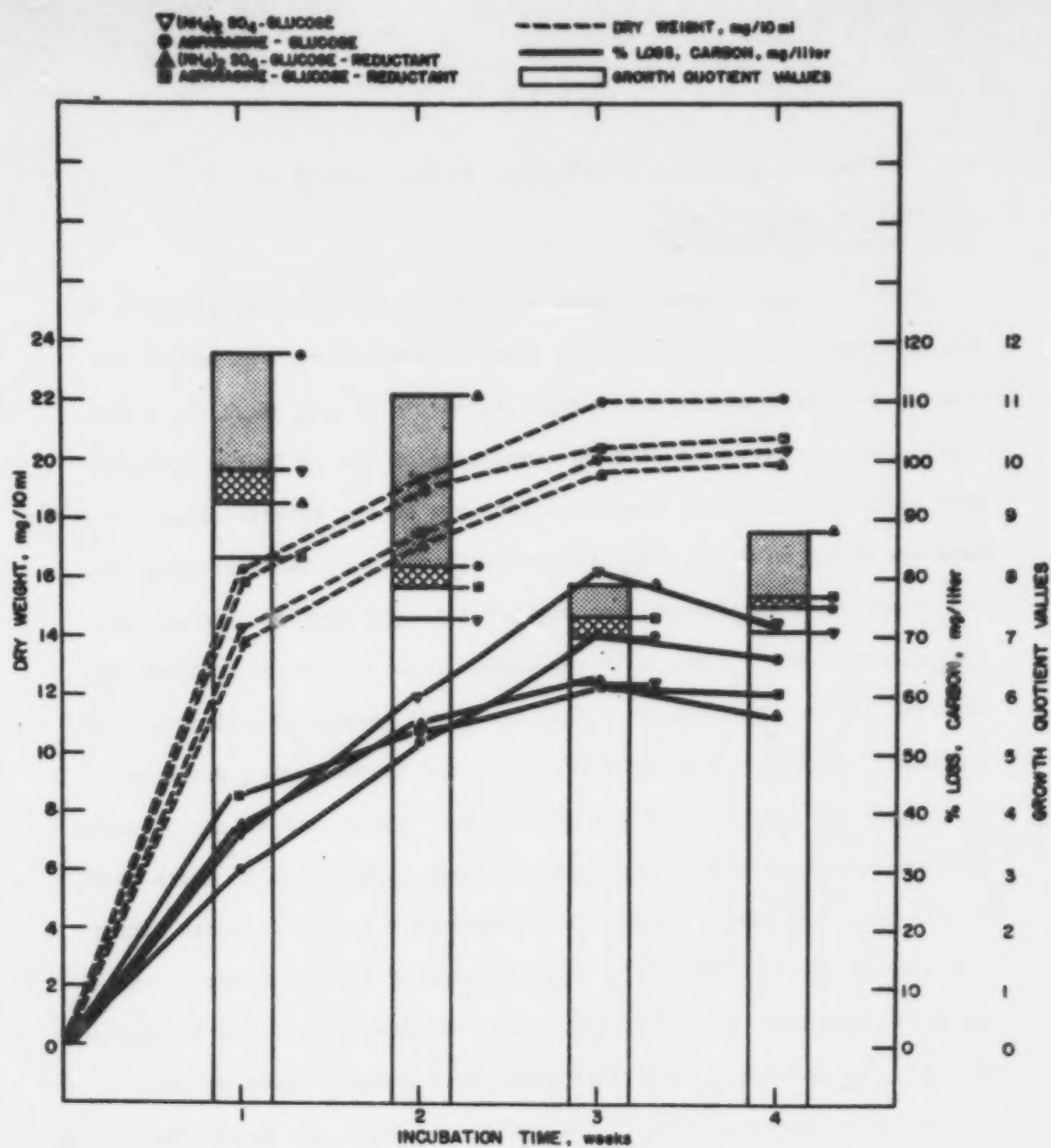
In all instances, an additional loss of total soluble organic carbon in media was observed during aerobic growth following the anaerobic pre-incubation, beyond the carbon loss demonstrated during growth in an atmosphere of 100% N_2 gas. Concomitant with this decrease of soluble organic carbon in the media incubated aerobically, following anaerobic pre-incubation, there was a rapid increase in the growth activity and development of the cells, followed by spore formation. These activities, which were usually absent in cultures grown anaerobically, were resumed when cultures were brought into contact with air.

A comparison of the economic efficiency of the 13 cultures grown anaerobically in media with organic and inorganic nitrogen source and without the reductant, for each week of incubation in the atmosphere of 100% N_2 gas was made. All 13 cultures grown in media with reductant yielded similar data. Economic efficiency data of cultures grown aerobically, following anaerobic pre-incubation were also obtained. In

our studies, the economic co-efficient is represented as

$$\frac{\text{mycelial dry weight, mg.}}{\text{total carbon consumed, mg}} \times 100.$$

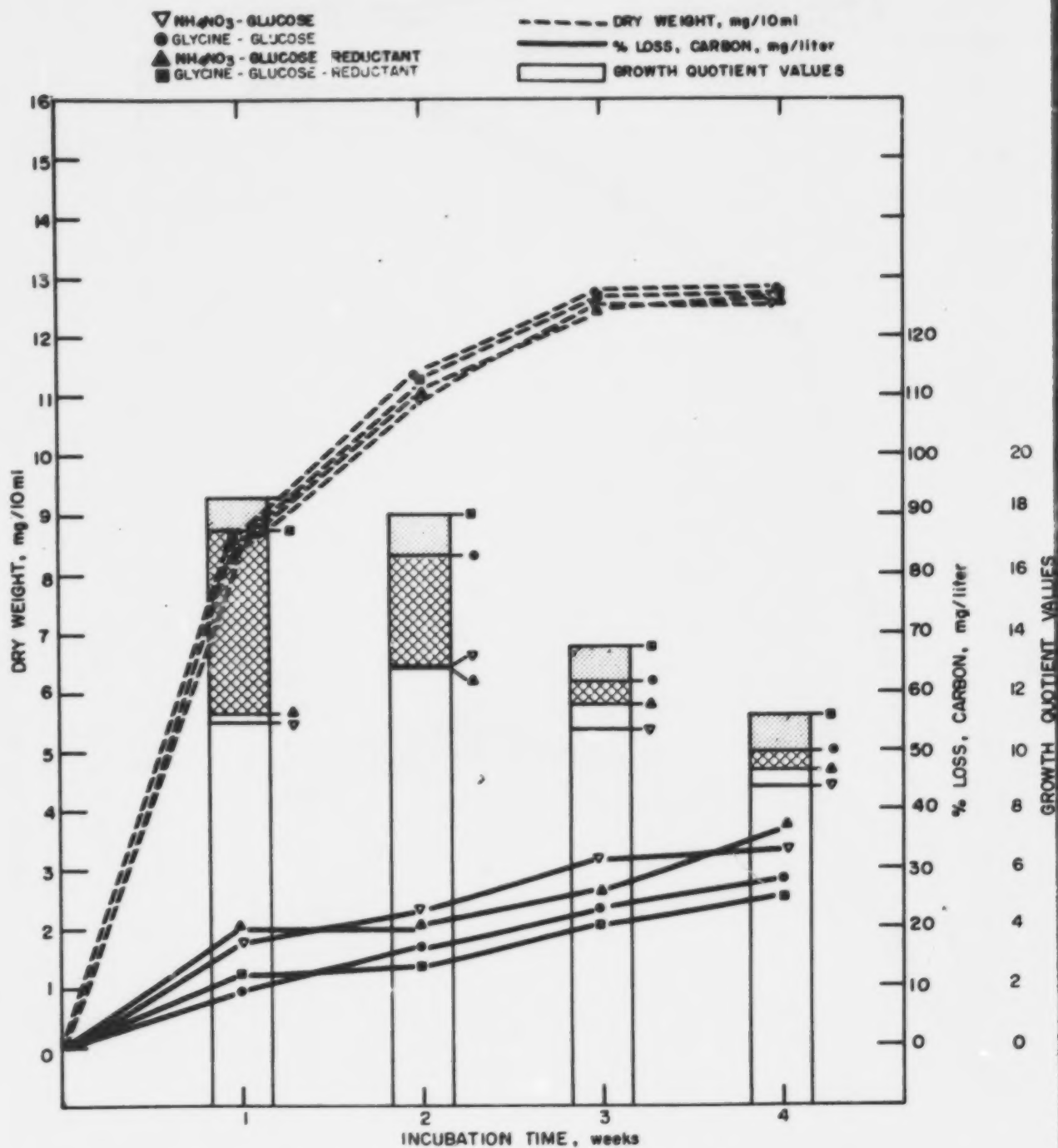
The increase of growth based on oven dry weight of mycelium in mg; the corresponding percent loss of total soluble organic carbon of the medium; and the economic efficiency values at the end of 1, 2, 3, and 4 weeks of incubation in 100% N₂ atmosphere of the 13 fungus cultures were plotted, and graphs are given for four of the species (Figs. 58-61). Data are given only for complete synthetic media, with or without the reductant and with inorganic or organic nitrogen sources. In all the 13 cultures a sharp rise, with variations from species to species, was observed in the growth curve within the first week of incubation, with a gradual tapering off of growth after 2 and 3 weeks and a subsequent plateau of the curve at the end of 4 weeks. The growth values in media with an organic nitrogen source are slightly higher than in media with an inorganic nitrogen source. This trend was observed in media either with or without the reductant. The addition of the reductant to the media was found to only slightly depress the growth for each incubation period. The species were divided into three groups, based on their growth curve characteristics. The species of the first group, Geotrichum candidum, Fusarium oxysporum and F. solani, exhibited growth curves of highest cell mass dry weight values, characterized by a very sharp rise in growth curve within the first week, followed by a gradual tapering off of growth activity throughout the second and third week of incubation, and a subsequent plateauing of the curve. The second group included, in



GEOTRICHUM CANDIDUM
FIGURE 58

Geotrichum candidum. Correlations of the increase in growth, based on oven dry weight of mycelium in mg; per cent of loss of the total soluble organic carbon per liter of the culture filtrate; and growth quotient values at the end of 1, 2, 3, and 4 weeks incubation in an atmosphere of 100% prepurified nitrogen gas.

Phialophora jeanselmei. Refer to Fig. 58 legend.

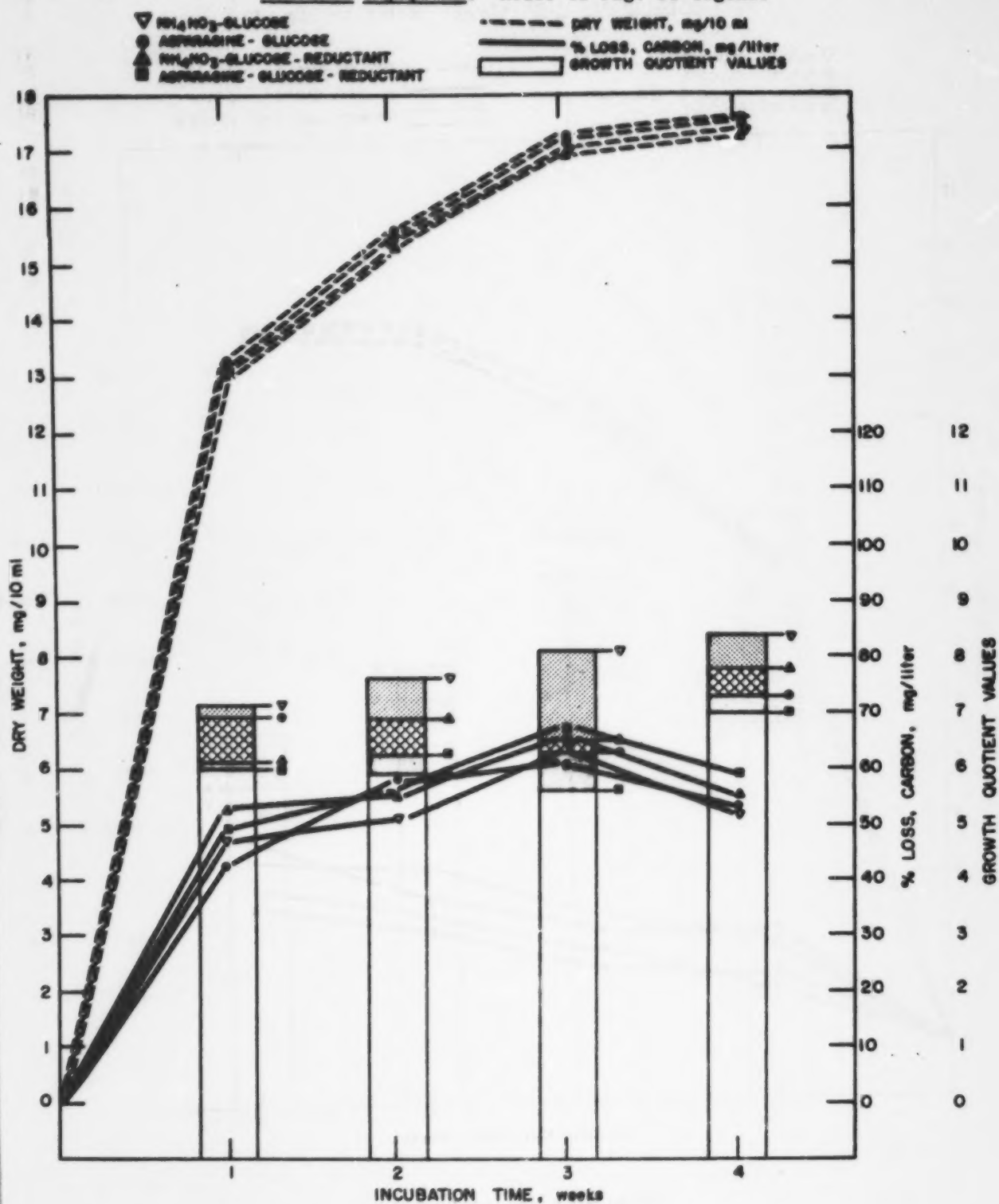


PHIALOPHORA JEANSELMEI

FIGURE 59

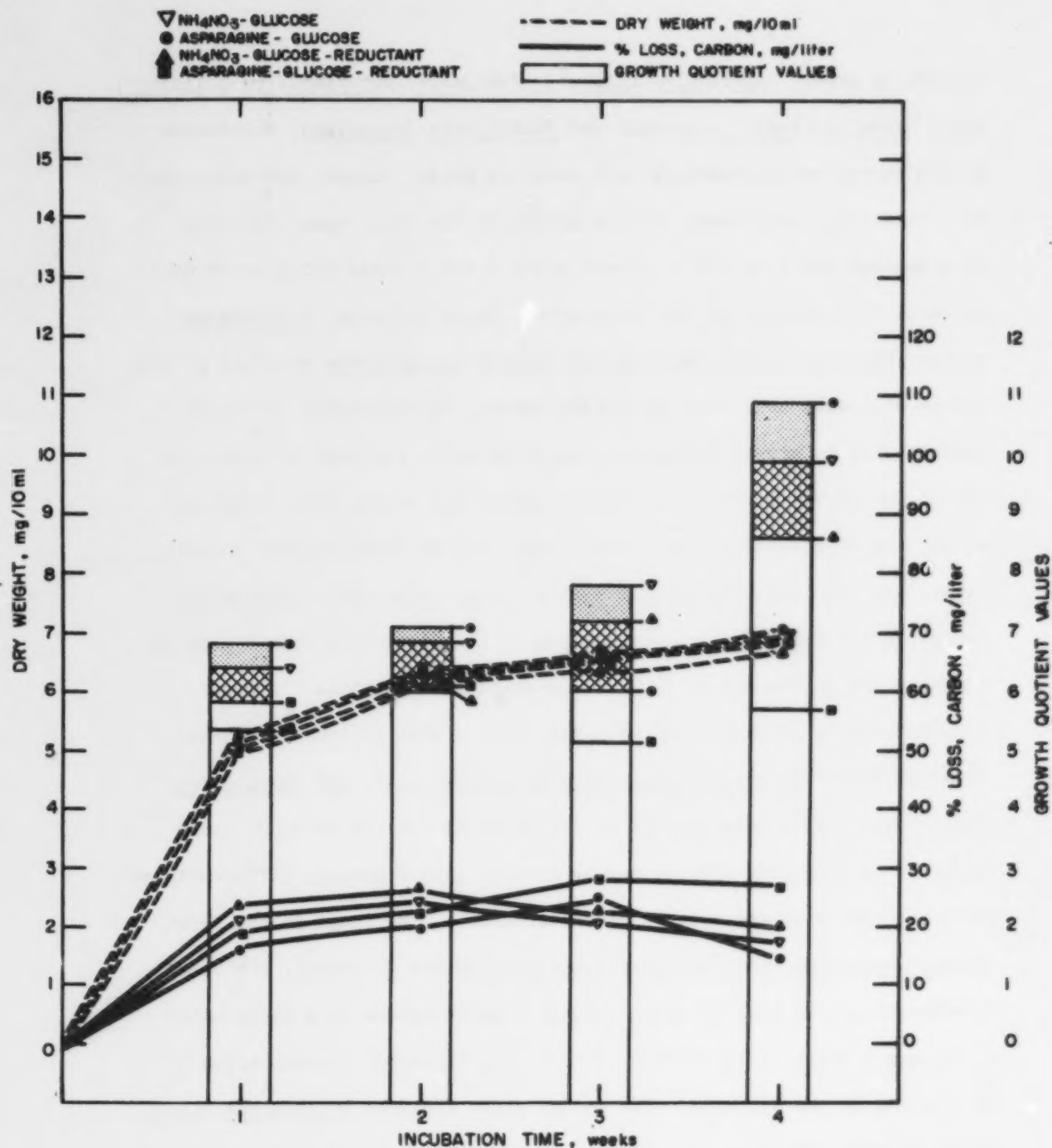
418b

Fusarium oxysporum. Refer to Fig. 58 legend.



FUSARIUM OXYSPORUM
FIGURE 60
418 c

Rhodotorula mucilaginosa. Refer to Fig. 58 legend.



RHODOTORULA MUCILAGINOSA

FIGURE 61

418d

descending order, Aspergillus niger, A. fumigatus, Aureobasidium pullulans, Mucor hiemalis, Penicillium spp. and Phialophora jeanselmei, which gave growth curves of intermediate cell mass dry weight values, characterized by a relatively less sharp rise in growth in the first week, followed by a gradual leveling off of growth after 2 and 3 weeks and a subsequent plateau. The species of the third group, Phoma herbarum, Trichoderma viride, Rhodotorula mucilaginosa and Candida parapsilosis produced growth curves of lowest cell mass dry weight values, characterized by a more gradual rise in growth throughout the first week, followed by levelling off of the growth activity at 2 and 3 weeks, and ending with a plateau at the end of 4 weeks. A relatively high loss of total organic soluble carbon was observed with Mucor hiemalis, Penicillium spp., Aspergillus fumigatus and Phoma herbarum at the end of the first week with a gradual tapering off at the end of 2, 3, and 4 weeks of incubation. A less pronounced loss of carbon in the media after 1 week of incubation was observed with Aspergillus niger, Candida parapsilosis, and Phialophora jeanselmei, with a leveling off of the curve at the end of 2, 3, and 4 weeks. The remaining species may be divided into 2 groups, differentiated from the others by their curve characteristics. The fungi in the first group, Geotrichum candidum, Fusarium oxysporum and F. solani, exhibited a relatively high loss of total soluble organic carbon from media after 1, 2, and 3 weeks of incubation, with a rise in carbon concentration at the end of the 4th week. The second group included Trichoderma viride, Aureobasidium pullulans, and Rhodotorula mucilaginosa, where a gradual loss of carbon from media was observed at the end of 1, 2, and 3 weeks

of incubation, with a similar rise in the carbon concentration at the end of the fourth week. The same relationship between organic and inorganic nitrogen sources and presence or absence of the reductant in the medium was observed as with carbon data, corresponding with growth curve results. The economic efficiency data for each culture are expressed in blocks, demonstrating the effects of the presence of the reductant and inorganic or organic nitrogen source in media at the end of 1, 2, 3, and 4 weeks of incubation.

Under 100% nitrogen gas, pH at the end of 4 weeks of growth varied less than 1.0 pH unit usually. Depending on the strain of the fungus species used, a few terminal pH levels were slightly less than the initial 3.4 or 4.0 in media without reductant or 4.0 with, most were slightly larger. In general, it was observed that there was a much lower drop in pH from the initial pH values in media with an inorganic nitrogen source under aerobic conditions of growth than during incubation in the atmosphere of N_2 gas. In media with an organic nitrogen source there was a much more pronounced rise in pH during aerobic incubation, than under anaerobic conditions of growth.

Anaerobic requirements. - The absence of the vitamins biotin and thiamine in the liquid synthetic media had a significant effect on the anaerobic growth and development of most of the fungal species, but did not appear to alter appreciably the anaerobic metabolic activity of these organisms. Results on the anaerobic growth requirements indicated that elimination of these vitamins from the media reduced the growth and developmental activity of all the species, possibly except Geotrichum candidum and the two Fusarium

species, from 20 to a maximum of 50% of the total anaerobic growth (based on dry weight cell mass values) in media supplemented with biotin and thiamine. The percentages of the loss of the final growth values, in media devoid of the two vitamins, varied from species to species. This reduction of the total growth activity indicated that, under anaerobic conditions, the synthesis of these vitamins by the organisms is impaired, and that the addition of preformed biotin and thiamine satisfied their growth factor requirements under anaerobic growth conditions. The response of each of the species to vitamin deficiency in the media varied, thus demonstrating differences in the capabilities of these fungi to synthesize vitamins in atmospheres lacking oxygen. The growth and development of Geotrichum candidum, Fusarium oxysporum and F. solani was apparently not appreciably affected by the omission of vitamins from the liquid media, indicating that either there was enough biotin and thiamine present in anaerobically growing cells (synthesized during aerobic growth of the inoculum) to support this growth, or that these fungi were able to synthesize these vitamins anaerobically or microaerophilically. The reducing agent, sodium formaldehyde sulfoxylate, was observed not to exert any effect on the anaerobic growth and development in media devoid of the two vitamins. The data expressing percent loss of total soluble organic carbon in culture filtrates, showed no significant changes in values from those obtained during anaerobic growth in media supplemented with biotin and thiamine. While the growth, multiplication and development of the cultures anaerobically, in media without the vitamins, was reduced, the utilization of glucose by these cultures was apparently unaffected.

Cultural characteristics - The cultures of the 13 fungi grown in an atmosphere of 100% nitrogen gas, were examined macroscopically at the end of the four week incubation period. The abnormalities of the culture characteristics in the media were noted, and comparisons of the type of growth were made for each culture grown in the complete, nitrogen-less carbohydrate-free, and basal synthetic media. These cultural characteristics were related to the normal growth patterns of each species grown aerobically. No observable effects on the gross morphology of the fungi grown either aerobically or anaerobically were apparent because of the presence of the reductant in the media. Table 107 summarizes the cultural characteristics of the anaerobic growth of the 13 species in complete media after 4 weeks of incubation in a 100% N₂ gas atmosphere compared with aerobic growth. In all instances, there was no evidence of sporulation activity in the cultures or pigmentation of growth, and the supernatant was found to be clear. The cultures are listed in the order of descending growth activity based on oven dry weight of tissue. Very good growth was demonstrated with Geotrichum candidum, Mucor hiemalis, Fusarium oxysporum and F. solani. Aspergillus niger, A. fumigatus, Penicillium spp., and Aureobasidium pullulans exhibited good growth, whereas Phialophora jeanselmei and Phoma herbarum gave fair growth. Sparse to poor growth was observed with Trichoderma viride, Rhodotorula mucilaginosa and Candida parapsilosis.

Discussion

The experimental data have demonstrated that the species of fungi used in this study could not only survive in an oxygen-free atmosphere

Table 107.

Cultural characteristics of the growth of fungi in complete liquid medium after incubation. (Listed in decreasing order of dry weight yielded under 100% nitrogen gas after 4 weeks growth.)

<u>Geotrichum candidum</u>	A. Four weeks under 100% nitrogen gas. B. One week under 100% nitrogen gas, four weeks on laboratory bench in air. A. Very good growth, white, with cottony consistency; surface, little on bottom of flask; clear supernatant; no sporulation observed. B. Very heavy growth, similar but with no apparent sporulation. A. Very good growth, white, with cottony consistency; surface, little throughout medium, none on bottom; clear supernatant; no sporulation. B. Heavy growth, cottony, superficial, with abundant sporulation. A. Very good growth, white, throughout the medium; no pigmentation observed; clear supernatant; no sporulation. B. Heavy growth, with inorganic N cottony, white, with pinkish-white pigmentation; with organic N pellular, white, with violet pigmentation on under surface of mat; in both med growth throughout medium; supernatant clear; abundant sporulation.
<u>Mucor biserialis</u>	A. Very good growth, white, throughout medium; no pigmentation; clear supernatant; no sporulation. B. Heavy growth, with inorganic N mycelial, straw colored supernatant and some cotton growth; with organic N white, pellular, with bluish-violet sporulation areas on surface and bluish-violet colored areas on under side of pellicle, brownish-orange supernatant, and some bottom growth; abundant sporulation.
<u>Fusarium oxysporum</u>	A. Good growth, white, with cottony consistency, throughout medium; no pigmentation; some submerged growth; clear supernatant; no sporulation. B. Heavy growth, black, composed of sporulating pellicle; supernatant clear in inorganic N, pale yellow in organic N; sporulation abundant.
<u>Aspergillus niger</u>	A. Good growth, white, on surface of liquid; some bottom growth; supernatant clear; no pigmentation; no sporulation. B. Very heavy growth, sporulating pellicle on surface; supernatant clear in inorganic N, yellow in organic N; sporulation green, heavy.
<u>Aspergillus fumigatus</u>	A. Good growth, white, with creamy consistency, throughout medium; supernatant clear; no pigmentation; no sporulation. B. Very heavy growth, sporulating surface mat; little bottom growth; supernatant straw yellow; sporulation heavy; dark green.
<u>Penicillium cyclopium</u> and <u>brevi-compactum</u>	A. Good growth, black, with pellicle-like consistency, gummy, gelatinous, mostly submerged; supernatant clear; no spore formation observed. B. Heavy growth, black, pellicular, gummy, gelatinous, some submerged growth; supernatant yellow; sporulation heavy.
<u>Aureobasidium pullulans</u>	

Phialophora jeanselmei

- A. Fair growth, dark green, clumpy, mostly submerged; poor growth on surface; clear supernatant; no spores observed.
 B. Heavy growth, dark, green, superficial, some submerged; clear supernatant; good sporulation.

Phoma herbarum

- A. Fair growth, grayish-green, pellicle-like; mostly submerged; clear supernatant; no sporulation observed.
 B. Good growth, in organic N surface growth with felt-like sporulation area, dark brown; growth mostly throughout medium; greenish-brown; in organic N surface growth a thick, yellowish-white pellicle with greenish-brown sporulation areas; supernatant straw-yellow; sporulation good.

Trichoderma viride

- A. Sparse growth, evenly distributed in medium, white; supernatant clear; no pigmentation or sporulation observed.
 B. Heavy growth, in inorganic N growth submerged, supernatant clear, some green sporulation; in organic N growth a heavy pellicle with cottony surface, supernatant yellow, good green sporulation.

Rhodotorula mucilaginosa

- A. Poor growth, very pale pinkish, at bottom of flask, supernatant clear.
 B. Heavy growth, at first superficial, then falling to bottom; supernatant clear, growth orange to red, creamy.

Candida parapsilosis

- A. Poor growth, white, with creamy consistency, at bottom of flask; no surface growth; clear supernatant.
 B. Good growth, creamy white, submerged; supernatant clear.

but were capable of limited development under 100% nitrogen in a reductant-containing medium. Although a certain amount of strain-specificity for tolerance to nitrogen gas, and variations in the extent of growth activity and in the rate of development of our cultures in an O₂-free atmosphere were observed, all of them were capable of developing to some extent under conditions of apparently complete anaerobiosis produced by a 100% N₂ gas atmosphere and the presence of a reductant in the medium.

Results of growth studies indicate that under anaerobic conditions growth was considerably poorer than under aerobic conditions. They do, however, demonstrate growth activity, more limited in some species than others, in an atmosphere of 100% N₂, and show a facultative anaerobic behavior of some of the organisms. The reductant added to the media did not significantly diminish the ability of the cultures to grow in an atmosphere of 100% N₂ gas, since the oven dry weight values of growth obtained from media with and without reductant were almost comparable. The reductant did not alter the growth ability of the organisms, nor was it shown to be toxic to the cells.

The results of soluble carbon residue tests indicate that, at least with some species, there is, in addition to significant growth activity, a relatively fair rate of metabolic activity. The organisms utilized glucose and a considerable portion of the molecule was retained for cell synthesis and growth, while the remaining portion was broken down with the resulting formation of residual materials. These findings demonstrate not only growth and cell synthesis but also an anaerobic respiration (fermentation) of glucose with production of unidentified byproducts.

The rapid loss of carbon, the increase of the rate, the extent of development of cultures, and the resumption of normal growth activity and sporulation, indicate that anaerobiosis did not have appreciable toxic effects on the culture.

Growth in control media without a carbohydrate source indicates that the fungus was using the carbon of the amino acids. Growth in control media without a defined nitrogen source indicates that there are impurities in the chemicals from which the media were prepared, excesses of nitrogen in the mycelium, or the mycelium was able to use some of the nitrogen from the atmosphere. However, it has not yet been shown satisfactorily that fungi of such a wide range of species are capable of using atmospheric N.

The low economic efficiency values suggest the presence of soluble carbon compounds in the medium, formed by the breakdown of glucose and a relatively less pronounced rate of incorporation of the glucose molecule as the energy source for the synthesis of the cellular constituents. A high economic coefficient implies greater utilization of the glucose molecule by the cells for synthesis of cellular components than for the production of soluble carbon byproducts. As an approximate index of the efficiency of carbon utilization, this economic coefficient was found satisfactory.

The results of the study on the influence of anaerobic pre-incubation on the growth and metabolic activity of the 13 cultures in air, indicate that anaerobiosis did not have any toxic effects on the cultures.

Results show that fungi isolated from digesting sludges and other

areas in sewage treatment systems where the oxygen supply is very limited, and those isolated from soil and litter as well as from acid mine drainage areas, are potentially tolerant to environmental conditions of extremely low O_2 , in spite of the fact that the cultures had been grown under aerobic conditions for greater or lesser periods of time after isolation. The ability of certain fungi not only to survive but to continue their development and growth activity under conditions of minute amounts of oxygen supply as well as anaerobic or microaerophilic conditions, enables them to metabolize some of the organic components of sewage and industrial wastes. The specific requirements of little or no oxygen for their growth and development, and tolerance to high N_2 levels and conditions of microaerophily or anaerobiosis can be advantageous in using the metabolic capacities of these fungi during anaerobic or microaerophilic waste and sewage treatment. This investigation has demonstrated that some of the common fungi of sewage and polluted water are capable of resisting conditions where the atmosphere is 100% nitrogen gas. These fungi not only tolerated such conditions, but grew and multiplied as indicated by the amounts of dry weight of cells recovered from growth solutions.

Specificity for the capability of growth and development of species in an atmosphere of 100% N_2 was observed, in that some of the species showed a remarkably lower tolerance to this condition of anaerobiosis or microaerophily while others developed fairly well. Of the fungi studied, Geotrichum candidum, Fusarium oxysporum, F. solani and Mucor hiemalis were not only most tolerant to conditions of total or nearly complete anaerobiosis, but were shown to be most capable in producing a high rate

and extent of growth under these conditions. The fungi of intermediate growth and developmental activity in this series of experiments were Aspergillus niger, A. fumigatus, Aureobasidium pullulans, Phialophora jeanselmei, Penicillium spp. and Trichoderma viride, while those with the lowest tolerance to the effects of N_2 gas, but still demonstrating limited growth, were Phoma herbarum, Rhodotorula mucilaginosa, and Candida parapsilosis. Those fungi tested demonstrated an absence of attenuation of developmental activity due to possible toxic effects of atmospheres of N_2 gas, tolerance to conditions of complete anaerobiosis (produced by the exclusion of O_2 and its replacement by N_2 gas), as well as an ability to grow and multiply anaerobically. This indicates that these and perhaps other sewage and soil fungi are capable of behaving as facultative anaerobes and can compete at least to some extent with strict anaerobic organisms in their growth and metabolic activities when such environmental conditions take dominance over others.

A requirement of the representative fungi for the vitamins biotin and thiamine under anaerobic or microaerophilic conditions has been demonstrated in our studies. The results on their anaerobic growth and metabolic activities in media devoid of these vitamins suggested that the absence of molecular oxygen limited the synthesis of these growth factors also, the vitamins had to be incorporated in the synthetic media to support the growth and multiplication of the fungi under the conditions of the experiments. The absence of thiamine and biotin from the media was found to reduce significantly the anaerobic or microaerophilic growth of most of the species. Slight reductions of growth, based on dry weights

of cell mass, were observed in Geotrichum candidum, Fusarium oxysporum and F. solani. Geotrichum candidum and the strains of Fusarium used demonstrated the ability to develop and multiply anaerobically in media devoid of biotin and thiamine. Therefore, it may be assumed that they are capable of limited synthesis of these vitamins under conditions of complete anaerobiosis or that a large supply of these vitamins was carried over in the inoculum used. The other species were observed to have an anaerobic requirement for biotin and thiamine.

Fungi have been observed to successfully colonize environments in which O_2 is very limited (Boutelje & Kiessling, 1964; Bulit & Louvet, 1960; Newcombe, 1960; Siepmann & Johnson, 1960; Thacker & Good, 1952; and Wair, 1957). The relatively stagnant aquatic habitat, heavily polluted waters, deeper areas of sludge beds, sludge digesters, and soil and forest litter produce microaerophilic conditions which may be partially or completely devoid of O_2 , and an atmosphere that may include toxic amounts of other gases. In these cases, fungi may metabolize organic compounds and may be capable of growth and development. Growth activity is the result of essential metabolic sequences that underlie the cultural development of the cells. Under anaerobic conditions, certain metabolic activities, fermentation, or anaerobic respiration are demonstrable without any evidence of concomitant growth activity. However, if growth and multiplication occur anaerobically they are obviously preceded by a sequence of metabolic reactions controlling growth activity. Our studies have demonstrated anaerobic or microaerophilic growth activity of the fungi tested. Tests have also shown fungi to be capable of maintaining

anaerobic respiration or fermentation of glucose probably through the Embden-Meyerhoff (Cochrane 1956) pathway, with the resulting production of byproducts including organic acids. Although many fungi ferment sugars anaerobically, which has no essential bearing on growth under anaerobic conditions, our studies show that many fungi have fermentative, as well as oxidative pathways of metabolism. The pathway of metabolism used by fungi is a function of the oxygen tension. The biochemical literature on fungi contains many examples of individual reaction products or products that involve the anaerobic EMP pathway (Gottlieb, 1963). The anaerobic EMP occurs in almost all species, and the entire pathway or parts of this system are active under aerobic conditions. But it has been established that reduced oxygen concentration or the absence of oxygen favors the operation of this pathway. The significant loss of the total soluble organic carbon in the media supporting anaerobic growth of fungi has provided evidence for the anaerobic respiration of glucose in an atmosphere of 100% N₂ gas, involving possibly some parts, if not the entire pathway, of the anaerobic EMP system.

SUMMARY

Thirteen representative species of fungi were studied to determine their ability to grow, multiply and develop anaerobically in an atmosphere of 100% nitrogen gas. The fungi were isolated in relatively large numbers from microhabitats devoid of measurable oxygen in sewage treatment systems, polluted waters, acid mine drainage areas, and litter and soil from forest regions.

To establish whether fungi grow as well as perform metabolic activities

in such habitats, liquid synthetic media containing either inorganic or organic nitrogen sources, with glucose as the carbon source and with or without the reducing agent, sodium formaldehyde sulfoxylate, were used. Incubation was effected in 25 ml cotton stoppered conical flasks, inoculated with shaker grown, blender treated and washed cells, stored in anaero-jars under 100% cylinder, pre-purified, nitrogen gas.

Growth and metabolic activities were measured at weekly intervals for 4 weeks, by oven dry weight determinations of cells collected on prewashed membrane filters, by analysis of the combustible residual total organic carbon in the culture filtrate, and by determinations of economic efficiency values.

The experimental results demonstrated that the organisms could tolerate and survive the apparently oxygen-free atmosphere. The organisms are capable of appreciable growth and development under 100% N_2 gas and exhibit significant metabolic activity under complete or nearly complete anaerobiosis. The species allowing the highest growth activity in a nitrogen gas atmosphere were Geotrichum candidum, Fusarium oxysporum, F. solani, and Mucor hiemalis. Those with intermediate growth and development were Aspergillus niger, A. fumigatus, Aureobasidium pullulans, Phialophora jeanselmei, Penicillium spp., and Trichoderma viride. The fungi most sensitive to a 100% N_2 gas atmosphere, but still capable of limited growth, were Phoma herbarum, Rhodotorula mucilaginosa and Candida parapsilosis.

All the species, with the exception of Geotrichum candidum, and the two species of Fusarium, demonstrated an anaerobic or microaerophilic requirement for the vitamins biotin and thiamine. The anaerobic growth

in media devoid of the two vitamins was considerably reduced, indicating an anaerobic or microaerophilic requirement for preformed vitamins. Geotrichum candidum and the species of Fusarium used had significant growth in media lacking biotin and thiamine. Therefore it may be concluded that these fungi are capable of synthesizing these vitamins under anaerobic or microaerophilic conditions.

XIII. Fungi in the Soil

A. Introduction

In preceding chapters it has been indicated that soil samples have been used rather extensively for determining some of the fungal populations of the streams, sewage treatment systems, and other habitats which have been examined for fungi in polluted waters and sewage. In all stream work, samples have been removed for study from the banks of streams where creek water kept the bank soil wet, in the earlier studies, and in later studies from sites beyond the influence of the stream at all times except unusually heavy flooding. The effect on fungal populations of atomized spent sulphite liquor was reported in one study. In attempts to compare clean and polluted areas, samples have been obtained from areas above what has been considered the source of pollution and even there the effects of previously unnoticed types of pollution were evident. In areas where streams carry acid mine drainage, soils adjacent to the streams show varying levels of iron content but this was not made evident until sampling had been completed and studies of iron content had been finished. No attempt was made to determine background levels and the effects of added quantities of polluting substances, although this type of information could probably be extracted from the data on a qualitative basis.

The only sewage treatment system dealing directly with soils was the waste stabilization pond system studied through four seasons. Here various soils as well as pollutinal materials were sampled and some idea obtained concerning the relation between undisturbed soil and soil to which organic matter was added regularly over a definite period of time. The reader is referred to Chapter VII.

Waksman (1917) and Goddard (1913) were among the first to definitely establish that there is a fungus population in the soil and that this population is useful in the biological processes continually going on within the soil. The recent symposium edited by Baker and Snyder (1965) again shows the importance of the fungal populations in the soil both as useful organisms and as potential pathogens of crop plants.

A number of soil samples have been processed in the study of fungi in this laboratory. Where these present material of interest they will be reported below. The more important of these studies is reported here almost as published.

B. Soil Mold Fungi Isolated from Recently Glaciated Soils in South-Eastern Alaska * - Introduction

The process of natural development of communities on terrain which has never before been populated has long interested biologists and soil scientists. Community changes involving the conspicuous organisms of many of these primary successions have now been fairly well described. But many details are still unknown, especially with regard to the microbiota, as to species composition, community organization and function, and rates of change. Growing interest in microbial ecology is evident in the recent books of Kelley (1950), Garrett (1956), and Williams and

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Spicer (1957).

Rapid recession of glaciers during the past two centuries in southeastern Alaska has made that region especially favorable for studies of vegetation development. The work of Cooper (1939 and earlier) on the development of vascular plant vegetation, and of Crocker & Major (1955), and Crocker & Dickson (1957) on soil development, have provided a firm foundation for studies of microbial succession.

In the hope of learning something about development of the soil mold fungus populations in the recent glacier deposits of known age, the writers in the Spring of 1955 planned a joint effort. Lawrence would collect fresh soil material from developing communities of differing ages ranging from zero at the ice edge to spruce (Picea sitchensis) forests 150 years old, and to much older hemlock (Tsuga heterophylla and T. mertensiana) forests, and Sphagnum moss and Carex muskegs on peat that had been accumulating for several thousand years; Cooke would carry on the culturing and identification at Cincinnati. Although this arrangement necessitated the unnatural conditions imposed by weeks of anaerobic storage and long distance transport, attempts were made to minimize these by refrigeration before and after air shipment. Chemistry of the culture medium and temperature conditions during culture were also very unlike those which occur in nature, but the artificial conditions were purposely provided to encourage the mold fungi, to discourage the bacteria, and to bring on reproductive stages so that identification could be accomplished in a reasonable time. In spite of inherent inadequacies, the results of these pioneer studies will at least provide data for preliminary consideration until laboratories become available

locally where more complete surveys can be carried out.

According to the list of Alaskan fungi drawn up by Miss Edith Cash (1953) no general surveys have been made of the soil fungi of this region. The present survey is considered a supplement to the extensive collections of fruiting material of the larger saprobic fungi made in 1952 by Sprague, and since reported by Cash (1954), Cooke (1955), and Sprague (1955). An ecological report on the 1952 study is now in preparation by Sprague and Lawrence.

Glacier History

Studies in north-western North America, Iceland, Scandinavia and the Alps have revealed that glaciers are generally in recession from positions of maximum extension attained in the seventeenth, eighteenth or nineteenth centuries. Evidence at hand indicates that glaciers were larger then than they had been for several centuries, and in some cases for at least several thousand years. In the subsequent recessions, extensive new surfaces have been laid bare upon which vegetation has gradually developed; the history of recession has in some of these regions been worked out in great detail, especially in south-eastern Alaska by Cooper (1937), Field (1947), Lawrence (1950) and Lawrence & Elson (1953). These new glaciated surfaces serve therefore as excellent areas in which processes and rates of community development can be studied. These studies have been summarized by Lawrence (1958).

The present work involves the terrain laid bare by two glacier systems - one, the great tidewater Muir Glacier and its tributaries in the Glacier Bay National Monument and the other, the Mendenhall Glacier outlet of the Juneau ice field, both in south-eastern Alaska.

The Glacier Bay area is unique: glacier recession there has been as much as 15 times more rapid than elsewhere, the recession amounting to 40 miles (64 km) during the past century and a half. This rapid recession has resulted in a relatively slow colonization by green plants; those with most mobile spores, such as lichens, mosses and Equisetum spp., and those with wind-borne seeds, such as especially Salix spp., Populus, Epilobium and Dryas, are the first to become established. Because Alnus and conifer seeds are less mobile, their seedlings are few and widely spaced in the early stages, delaying development of dense shrub thicket so that approximately 100 years have been required for closure of the first evergreen forest canopy. In the Glacier Bay area several species of Dryas, which are important mat-forming, root-nodule-bearing woody plants, usually form an extensive ground cover before Alnus-Salix shrub thickets develop.

In the Mendenhall Valley, on the other hand, glacier recession has been much less extensive amounting to only 2 miles (3.2 km) in the past 2 centuries, so that seed sources have always been close at hand and the seedling conifers have become established promptly after ice recession, often within 5 years. As a result, evergreen forest canopy closure has occurred in a half century; Dryas, furthermore, has been completely absent. There are thus notable differences between the Glacier Bay and Mendenhall study areas, and these differences are reflected in the fungus surveys reported here.

Beyond the terrain overridden by ice a few centuries ago occur surfaces of much greater age which have been vegetated for as much as

10,000 years since general glaciation. The steepest of these older surfaces are forested with hemlocks (Tsuga heterophylla and T. mertensiana) and some of the lower gentler slopes with coast pine (Pinus contorta). On the relatively level surfaces the forests have been displaced gradually by Sphagnum-Carex muskegs as water logged peat has accumulated; these subsequently have become dotted with pit ponds in mild, humid, south-eastern Alaska. The Eagle Glacier muskeg, which lies 12 miles (12.9 km) north-west of the Mendenhall area and a few hundred feet down-valley from the eighteenth century terminal moraine of Eagle Glacier, is a fine undisturbed example of these older surfaces in a very late vegetation stage. Samples of peat from the present surface and from sub-surface levels were collected there for comparison with the younger soils from the Glacier Bay and Mendenhall areas.

Soil Development

Glacier ice lies on a heterogeneous parent material which includes many rock fragments. After the insulating blanket of ice has gone, the coarser rock fragments are gradually brought to the surface, as a result of freezing and thawing, where they form a pavement of stones. Immediately below the stone pavement lies a layer predominantly of sand, silt and clay. With the development of vegetation on the pavement of stones, litter (L) accumulates and freezing and thawing are prevented by the organic insulation. As this organic blanket thickens, rate of accumulation exceeds that of decay, resulting in the development of a fermentation layer (F) and later, below that, a layer of humus (H), all superimposed upon the stone pavement. The mineral profile gradually becomes weakly podsolized. In many areas a layer of mosses develops

on top of the litter, at first consisting of forest mosses and later of species of Sphagnum, so after several thousand years the forest floor becomes waterlogged, the trees gradually die out, a Sphagnum-Carex muskeg vegetation develops as described by Zach (1950) and Lawrence (1958), and the peat layer attains depths of several meters.

Sampling Method

Samples from which the fungi listed below were isolated were obtained by D. B. and Elizabeth G. Lawrence and R. E. Schoenike in July and August 1955 while on an expedition supported by the Graduate School of the University of Minnesota and the American Geographical Society.

The field sampling began at the youngest soil sites and proceeded chronologically to older and older surfaces to avoid artificially inoculating the younger surfaces with organisms from the older communities. An attempt was made to obtain material from the organic portion of the profile in the litter (L), fermentation (F) and humus (H) layers, from the predominantly mineral portion in the upper 2 in. (5 cm.), and at the 6 in. (15 cm) level below the mineral surface. Each soil collection was made within a circle of approximately 12 sq. in. (77.6 sq. cm.) with a knife blade that had been flame sterilized and allowed to cool to air temperature. For subsurface samples, a pit was dug with a shovel brought into biological harmony with the soil at a given collecting station by scrubbing thrusts (about 10) into an adjacent portion of the soil. The sample was placed in a previously unopened plastic bottle ("Plastainer") holding 60 ml of material. After filling and capping with the polyethylene stopper, care was taken to keep the bottle

cool and away from direct sunlight. The filled bottles were refrigerated after collection, shipped by air parcel post to Cincinnati, and again refrigerated at approximately 5°C until the samples could be plated on culture medium. The history of the samples is presented in Table 108.

To test the containers for freedom from contamination, four unused bottles were opened in the laboratory at Cincinnati, and a film of medium used in isolation of fungi from the samples was allowed to solidify on the inner surface. This medium had been stored in 10 ml portions in a water bath at 45°C. It cooled quickly as aureomycin was added and as the medium was poured into the bottles. It is thought that the treatment had little or no thermal inhibitory effect on possible fungal contaminants. A single mold colony, different from any fungus isolated from the samples, developed from one bottle. On the basis of this test, the bottles were considered to be relatively sterile, contributing no significant colonies to the survey.

The plating technique has been described in an earlier chapter.

Description of Sampling Sites

Glacier Bay area

Station 1 - Surface of ice, 3 ft. (0.9 m) from melting edge, east shore, head of Muir Inlet. Surface age zero.

Station 2 - Surface of fresh till, 20 ft (6.1 m) from ice edge, near Station 1. No visible plant cover. Age about 2 months.

Station 5 - Till with complete moss cover, 1/4 mile (0.4 km) south from ice edge, northeast shore of Anchorage Cove, east shore of Muir Inlet. Plants included scattered Salix spp. (mainly S. sitchensis) and Populus

Table 108. History of collection, transport, storage and culture of samples in 1955.

Sample source	Field Station Numbers	Collected	Shipped	Received	Plated	Counted
Glacier Bay	1-23	11 Aug.	22 Aug.	24 Aug.	20 Oct.	27 Oct.
Glacier Bay	26-35	11 Aug.	22 Aug.	24 Aug.	12 Nov.	19 Nov.
Glacier Bay	39	19 Aug.	22 Aug.	24 Aug.	12 Nov.	19 Nov.
Mendenhall Glacier	48-53	26 Aug.	27 Aug.	30 Aug.	12 Nov.	19 Nov.
Mendenhall Glacier	58-81	26 Aug.	27 Aug.	30 Aug.	28 Nov.	5 Dec.
Mendenhall Glacier	86-93	26 Aug.	27 Aug.	30 Aug.	2 Feb. 1956	12 Feb. 1956
Eagle Glacier musing	101-105	4 July	5 July	7 July	7 July	14 July

trichocarpe, 7 years old, occasional Anaphalis margaritacea, and one small Dryas drummondii. Sampled at surface and 1/2 inch (1.3 cm.) in the mineral material. Age 8 years.

Station 23 - Under center of Dryas drummondii mat 10 ft (3.0 m) in diameter and 16 or 17 years old, with numerous root nodules, southeast of Anchorage Cove, east shore of Muir Inlet. Four Salix spp., up to 2 ft. (0.6 m.) tall, occurred in the mat. Sampled L, 1 in (2.5 cm.) and 6 in (15 cm). Age about 20 years.

Station 26 - Beneath Alnus crispa var. sinuata about 10 years old, 6 ft (1.8 m) tall, northeast of Goose Cove, east shore of Muir Inlet. Populus also present, the oldest 21 years. No Dryas was present before the Alnus.--Sampled L, 1 in. and 6 in. Age about 22 years.

Station 32 - Under Alnus about 14 years old, 12 ft (3.6 m) tall, 100 ft (30 m) south of Goose Cove Creek. A continuous Dryas mat was present here before the Alnus. Sampled L, 1 in. and 6 in. Age about 34 years.

Station 35 - Under Alnus and Populus with no living moss or herbaceous ground cover, shore of the main bay west of the north end of Willoughby Island. A heavy Alnus leaf mould up to 2 in. (5 cm.) thick with many fruiting agarics covered the till-pebble surface. Plants included old sprawling Alnus thicket 20-25 ft (6.0-7.5 m) tall with stems 5 in (7.6 cm.) in diameter at the base, and an overstorey canopy of Populus 35-40 ft (10.5-12.1 m) tall and up to 9.3 in (23.6 cm) in diameter at breast height (DBH). Spruces (Picea sitchensis) nearby 30-40 ft (9-12 m) tall, mostly 5-7 in. (12.7-17.8 cm) dbh, one 15 in (38.1 cm)

dbh, did not form cover or litter in area sampled. Sampled L, F, H, and 6 in. Age 75-90 years.

Station 39 - Gently rolling till in Picea forest, south side of Lester Island, Bartlett Cove. Closed canopy formed of Picea 21-22 in. (53-56 cm) dbh and 110 ft. (33.4m) maximum height, Populus 16-17 in. (41-43 cm) dbh, and Tsuga heterophylla 4-6 in. (10-15 cm) dbh. Understorey sparse, park-like; shrubs up to 4 ft. (1.2 m) tall included Rubus spectabilis, Sambucus racemosa, Oplopanax horridus, Vaccinium ovalifolium and occasional Ribes sp. Ground cover included 5 moss species and 12-15 herbaceous species, Rubus pedatus being the commonest. The total depth of L, F, and H layers was 8-12 in (20-30 cm). Sampled L, F, H, and 6 in. Age about 150 years.

Mendenhall Glacier area

All samples except at Station 93 were collected from the east side of the valley floor, southward from the ice edge.

Station 48 - Surface of till layer 1 in thick on ice at southeast edge of glacier front. Age zero.

Station 53 - Edge of young moraine. Plants included complete moss cover, patches of the lichen Stereocaulon, occasional Picea and Tsuga seedlings, Alnus to 12 in. (30 cm.) tall, Salix spp., Equisetum sp., Epilobium sp. and grasses. Sampled surface 1 in. and 6 in. Age 10 years.

Station 58 - Young Alnus and Salix thicket, surface litter mostly of Alnus leaves. Plants included rapidly growing Alnus 32 in. (83 cm) tall, Picea and Tsuga seedlings, several Salix, a plant of Cassiope sp., mosses and lichens. Sampled L, 1 in. and 6 in. Age 13 years.

Station 72 - Dense Alnus and Salix thicket. Litter mostly of Alnus

and Salix leaves. Plants included Alnus up to 16 ft. (4.8 m) tall and 4 in. (10 cm) in basal diameter, Salix sitchensis up to 25 ft. (7.6 m) tall and 4 in dbh, S. barclayi and S. alaxensis, numerous young Picea and occasional Tsuga, mosses, a Calamagrostis and occasional fruiting specimens of Aruncus sp. Sampled L, F, combination of H and first inch of mineralsoil, and 6 in. including some roots. Age 23 years.

Station 81 - Young Picea forest overtopping Alnus thicket. Forest floor was 3 in (7.5 cm) thick, and moss cover was present. Plants included Picea up to 28 ft (8.5 m) tall and 7 in (17.8 cm) dbh, Tsuga 6 ft (1.8 m) tall and 1/4 in (0.6 cm) dbh, Alnus stems up to 18 ft (5.5m) long and 6 in. basal diameter, Salix up to 15 ft. (4.6m) tall common, occasional Populus up to 28 ft (8.5m) tall and 7.5 in (18 cm) dbh. Sampled L, F, H, 1 in and 6 in. Age 61 years.

Station 86 - Crest of terminal moraine 10 ft above outwash surface. Dense moss ground cover and forest floor totaled 5-6 in. deep. Plants included main canopy of Picea approximately 100 ft (30.4 m) tall and up to 19 in. (48 cm) dbh, Tsuga 50-60 ft (15-18 m) tall and up to 8 in (20 cm) dbh, occasional old Populus up to 40 ft. (12 m) tall with broken tops and 15 in. (38 cm.) dbh, remnants of living Alnus shrubs up to 8 ft. (2.4 m) tall, Lycopodium sp. and Cornus canadensis common. Sampled L, F, H, 1 in and 6 in. Age 160 years.

Station 93 - Wet soil on late-Glacial moraine slope on west side of valley in ancient Tsuga forest several generations old. General moss cover was complete and deep, litter rarely seen. Soil was dark colored, upper 9 in (23 cm) forest peat, then predominantly mineral soil containing pebbles down to rocky pavement beginning at 22 in. (56 cm). Tree canopy

included old, slowly growing Tsuga in poor condition with trunks to 18 in. (45 cm) dbh, many dead and fallen, and a few slowly growing Picea up to 20 in. (51 cm) dbh and over 600 years old, rooted on logs of a previous generation. Common shrubs included Menziesia and Vaccinium. Herbs included Cornus canadensis, Coptis asplenifolia, Rubus pedatus, ferns and Lysichitum americanum. Sampled L beneath living moss cover, F, H, 2 in. and 6 in. Age at least 1000 years, and probably about 6300 years as indicated by Lamont Laboratory radiocarbon dating (Heusser, personal communication to Lawrence); the latter is the period of time since this land rose from the sea.

Eagle Glacier Muskeg

A series of samples was collected from the Eagle Glacier muskeg overlying late-Glacial till a few hundred feet down the valley from the eighteenth-century terminal moraine of Eagle Glacier, and 12 miles (19.2 km) northwest of the Mendenhall area, for comparison with the much more recently glaciated surfaces listed above.

Sample 101 - Living Sphagnum on the surface of the muskeg.

Sample 102 - Living Sphagnum at edge of a pit pond 3 ft in diameter.

Sample 103 - Surface of a black patch between living Sphagnum plants.

Sample 104 - Sub-surface Sphagnum peat collected at a depth of 1.5 m.

Sample 105 - Sub-surface Sphagnum peat from the base of the organic deposit at a depth of 2.5 m.

Samples 101, 102, and 103 are comparable with litter layer samples at the other stations. Samples 104 and 105 are comparable with fermentation layer samples. They were collected by C. J. Heusser with a Hiller peat auger.

Results and Discussion

Presentation of Data

To facilitate comparison of kinds and numbers of mold colonies cultured from the samples taken at the various field stations and soil layers, lists are given in Tables/ ^{109, 110, 111, and 112.} Information on the fungi found in greatest average abundance in the combined cultures from all soil layers at a given station is summarized in Tables 113 and 114, making possible a uniform method of comparison for the stations without influence from sample numbers, which ranged from 1 to 5 at the different stations, depending principally on the number of soil layers present. ^{113 114} It seems clear in Tables / and / that the average numbers of colonies derived from the soils of both Glacier Bay and Mendenhall areas are greatest in the Alnus stage cultures. More evidence is presented in ¹¹⁵ Table / supporting the idea that there is a casual relation between mold fungus activity and availability of nitrogen, symbiotically fixed in the Alnus stage (Bond, 1955) and quite possibly in the Dryas stage also ¹¹⁶ (Schoenike, 1959). Table / gives lists of species that were apparently restricted in their distribution to one of the three localities, or to the organic or mineral portion of the profile. Additional and more intensive sampling and culturing would very likely show that many of these fungus species occur in one of the other areas or levels as well.

In the histograms of Figures 62 and 63, values are given for the series of stations from which samples were taken, arranged from left to right in order of increasing surface age and developmental stage of vegetation. The numbers of colonies of fungi per gram dry weight found in each soil

Table 109. Species of fungi obtained from the Alaska soil samples.
G - Glacier Bay, M - Mendenhall Glacier, E - Eagle Glacier

Horizon	A ₀₀	A ₀	A ₀	A ₁	B ₁	B ₂	C ₁
Basic Number of Samples	18	9	4	18	13	8	14
Composite list of fungi:							
<u>Alternaria tenuis</u>	G	E		GM	M		G
<u>Aspergillus flavus</u>	M		M	M	M		M
<u>niger</u>	G				G		M
<u>sydowii</u>	M		M	M	GM		M
<u>ustus</u>	G						
<u>versicolor</u>	G	G E	M	GM	GM	GM	GM
<u>Cephalosporium coremioides</u>	G	G		GM	GM	GM	GM
<u>Cephalosporium spp.</u>			M	G	G		G
<u>Cladosporium cladosporioides</u>	GME	GME	GM	GM	GM	GM	GM
<u>Coniothyrium fuckelii</u>							M
<u>Dematiaceae spp.</u>	G E	G		G	G	GM	G
<u>Fusarium roseum</u>				G			
<u>Geotrichum candidum</u>	G			G			G
<u>Phialophora jeanselmei</u>				G		M	G
<u>Moniliaceae spp.</u>	GME	GME	GM	GM	GM	GM	GM
<u>Mucor angulisporus</u>	E	M	M	M			
<u>fragilis</u>		M					
<u>hiemalis</u>	GM	GM	M	GM	GM		GM
<u>hiemalis</u> sect. n.sp.	GM	GM	M	GM	GM		
<u>mucedo</u>			M				
<u>Oospora lupini</u>					M		
<u>Paecilomyces variotii</u>					M		
<u>Penicillium aculeatum</u>					G		
<u>chrysogenum</u>							G
nr. <u>fellutanum</u>						M	G
<u>herquei</u>			M				M
<u>implicatum</u>							G
<u>kapuscinskii</u>	M		M	M		M	G
<u>lanosum</u>			G	G	G		G
<u>lilacinum</u>	GM	GM	M	GM	GM	GM	GM
<u>ochro-chloron</u>	E				M	M	GM
<u>oxalicum</u>	GM			GM	GM		GM
<u>piceum</u>		E					
<u>purpureogenum</u>				M			
<u>spinulosum</u>		M	M		G		
<u>thomii</u>	G	GM		GM	G	G	G
<u>variabile</u>	M		M		M		
<u>velutinum</u>				M			M
<u>urticae</u>							G
<u>Penicillium spp.</u>	G E		M	G		M	GM
<u>Phialophora fastigiata</u>	M						
<u>Phoma spp.</u>	G		G	G	G		GM
<u>Aureobasidium pullulans</u>		G		M		G	GM
<u>Pythium sp.</u>	M	M	M				
<u>Rhodotorula glutinis</u>		M	M	M		M	G
<u>Scopulariopsis sp.</u>	E						
<u>Sepedonium sp.</u>						M	
<u>Sporotrichum sp.</u>							G
<u>Trichoderma viride</u>	GM	GM	GM	GM	G	G	GM
<u>White Yeasts</u>	GM	ME	M	GM		GM	G
Species: Glacier Bay	18	11	4	20	18	10	27
Mendenhall	14	13	20	18	15	14	18
Eagle	7	6					
Total	27	19	21	28	24	17	33

Table 110. Glacier Bay: Continuity of the fungus species through the successional stages: all soil levels combined.
Values show total number of colonies in cultures, corrected to dilutions of 1:100

Station Number and Vegetation Stage	1 Ice Surface	2 Fresh Till	5 Moss	23 Dryas Mat	26 Young Alnus without Dryas	32 Dense Alnus with dead Dryas	35 Populus	39 Picea sitka- ensis
Surface age, years	00	1/6	8	20	22	34	75-90	150
<i>Penicillium chrysogenum</i>	10		10					
<i>Penicillium roquei</i>			10					
<i>Penicillium urticae</i>			10					
<i>Phoma</i> spp.			540	52		54		
<i>Dematiaceae</i> spp.			50				90	20
<i>Aspergillus versicolor</i>			20				12	10
<i>Aureobasidium pullulans</i>			30				10	
<i>Sporotrichum</i> sp.			30	20	1			
<i>Aspergillus niger</i>				10				
<i>Penicillium implicatum</i>				2				
<i>Penicillium griseolum</i>				10				
<i>Rhodotorula glutinis</i>				4				
<i>Neurospora</i> sect. sp.n.				26				
<i>Penicillium thomii</i>				965			2010	20
<i>Neurospora</i>					167	524	120	30
<i>Geotrichum candidum</i>					297	619	30	
<i>Trichoderma viride</i>					39	32	20	
White yeasts					31	755	10	260
<i>Aspergillus utrum</i>					42			
<i>Penicillium fellutanum</i>					2	10		
<i>Alternaria tenuis</i>						1	10	
<i>Penicillium lanosum</i>						1	10	
<i>Phialophora festucae</i>						261		
<i>Opisthoxylum cornuoides</i>						3		10
<i>Neurulaeae</i>	30		250	950	4444	1756	200	270
<i>Cladogonium cladogonioides</i>	20	30	10	34	40	3375	4061	605
<i>Penicillium caninum</i>		10	30	12	1	1	24	70
						10	10	
	3	2	11	11	11	14	15	10
	60	40	1000	2085	5065	7392	6615	1425
	1	1	3	3	3	3	4	4

Total per Station
Colonies per station
Number of samples

Table 111. Mendenhall Glacier: Continuity of fungus species through the successional stages, all soil levels combined. Values show total number of colonies in cultures corrected to dilutions of 1:100.

Station number and vegetation stage	48 Ice Surface	53 Pioneer Moss	58 Young Alnus	72 Dense Alnus and Salix	81 Trans- ition Picea sitkensis Populus 61	86 Picea sitkensis forest	93 Old Tsuga forest
Surface age, years	0	10	12	23	160	+1000	
<i>Penicillium purpureogenum</i>	1						
<i>Cephalosporium cornuoides</i>	2	11					
<i>Aureobasidium pullulans</i>		1					
<i>Aspergillus versicolor</i>		1		20			
<i>Penicillium oxalicum</i>			30				
<i>Phialophora fastigiata</i>			600				
<i>Paecilomyces varioti</i>			10				
<i>Oospora lupini</i>			30				
<i>Coniothyrium fuchelii</i>			10				
<i>Penicillium valutinum</i>			10				
<i>Phoma</i> spp.			10				
<i>Aspergillus flavus</i>			10	10			
<i>Aspergillus sydowii</i>			180	10			
<i>Penicillium ochro-chloron</i>			10	10			
White Yeasts			110	60	250		10 6500
<i>Penicillium variabile</i>			10				20
<i>Penicillium</i> spp.			60				
<i>Dematiaceae</i> spp.				450			
<i>Cephalosporium</i> sp.					10		
<i>Penicillium</i> nr. <i>fellutanum</i>					2500		
<i>Mucor fragilis</i>					10		
<i>Pythium</i> sp.					10		
<i>Penicillium karyocinastii</i>					10		
<i>Rhodotorula glutinis</i>					2670	870	
<i>Sepedonium</i> sp.					150	380	
<i>Mucor ananiasporus</i>				320	60	10	20
<i>Penicillium spinulosum</i>					450	20	
<i>Penicillium barqueti</i>						20	
<i>Mucor mucedo</i>						10	
<i>Penicillium thomii</i>						40	
<i>Phialophora jeanneanae</i>							30

Table 111. p. 2.

<u>Cladosporium cladosporioides</u>	11	120	20	30	20	60
<u>Trichoderma viride</u>	1	40	200	160	450	190
<u>Penicillium lilacinum</u>	1	1	10	80	960	150
<u>Moniliaceae spp.</u>	10	230	10870	9825	5530	710
<u>Mucor hiemalis</u> sect. n.sp.			540	1935	730	20
<u>Mucor hiemalis</u>		290	40	40	3010	620
Taxa per Station	7	18	13	15	13	11
Colonies per Station	27	1761	12560	18180	12090	8020
Number of samples	1	3	4	5	5	5

Table 112. Fungi Isolated from Eagle Glacier Mushing Samples.

Sampling Site Sample Numbers	Surface 101	102	103	Subsurface 104	105
<u>Penicillium spp.</u>	x	x	x		
<u>Moniliaceae spp.</u>	x	x	x	x	x
<u>Scopulariopsis sp.</u>		x			
<u>Nucor angulisporus</u>		x			
<u>Demiticeae spp.</u>		x			
<u>Cladosporium cladosporioides</u>		x		x	
<u>Penicillium ochro-chloron</u>			x		
<u>Penicillium piceum</u>				x	
<u>Alternaria tenuis</u>				x	x
<u>Aspergillus versicolor</u>					x
<u>White Yeast</u>					
Total colonies per ml on 1:100 basis	130	380	700	30	40
pH of 1:10 suspension	4.3	4.0	4.1	3.6	4.1

Note: Insufficient material sampled for dry weight determinations.

Table 113. Fungus succession: Species important in various vegetation stages, that is, 10 or more colonies at any station. Importance values at any one station represent average numbers of colonies per sample for all soil levels combined: *** 1000 or over, ** 100-999, + 10-99, . = also present.

Vegetation State	Ice surface	Fresh Till	Pioneer Mosses	Dryas Mat	Young Alnus	Dense Alnus	Trans- ition Populus	Picea Forest	Tsuga Forest	Muskeg*
<i>Penicillium chrysogenum</i>	+							+		
<i>Cladosporium cladosporioides</i>	+	+	+	+	+	++	+	++		
Moniliaceae spp.	+		+	+		+				
<i>Penicillium oxalicum</i>		+	+	+		+				
<i>Phoma</i> spp.			++	+			+			
Dematiaceae spp.			+							
<i>Aureobasidium pullulans</i>			+							
<i>Sporotrichum</i> sp.			+							
<i>Penicillium thomii</i>			+	++		++	++			
<i>Mucor hiemalis</i> Sect. n.sp.				+		+				
<i>Mucor hiemalis</i>					+					
<i>Ooetrichum candidum</i>					+	++				
<i>Trichoderma viride</i>					+	++		+		
<i>Aspergillus niger</i>						+				
<i>Penicillium lanosum</i>						+				
<i>Cephalosporium cornuoides</i>						+	+	+		
<i>Cladosporium cladosporioides</i>	+		+							
<i>Trichoderma viride</i>	+					+		+	+	
<i>Penicillium lilacinum</i>	+					++	+	++	+	
Moniliaceae spp.	+		+			++	++	++	++	
<i>Mucor hiemalis</i>			+			+		++		
<i>Mucor hiemalis</i> Sect. sp.n.						++	+	++		
<i>Phialophora festigata</i>					++					
<i>Oospora lupini</i>					+					
<i>Coniothyrium fuscum</i>					+					
<i>Aspergillus nidulans</i>					+					
<i>Penicillium oxalicum</i>					+	++		++		
<i>Penicillium</i> spp.					+	+		++		
White yeasts					+	+			++	
<i>Penicillium</i> nr. <i>fellutanum</i>						+	+	++		
<i>Penicillium lanosum</i>							+			
<i>Beudanticum</i> sp.							+			
<i>Rhodotorula glutinis</i>							+			
<i>Mucor sanguinolentus</i>						+				

* Numerical data not truly comparable with the other stations.

Table 114. Summary of fungus succession data from Table XIII-6.

Vegetation Stage	Ice S	Fresh	Moss	Dryas	Young Alnus	Dense Alnus	Trans- ition	Picea	Tsuga	Muskeg
Glacier Bay										
.										
+	3	2	1	3	2	1	7	3		
++			5	2	6	5	3	3		
+++			1	2		4	1	1		
+++					1	1	1			
Total	3	2	7	7	9	11	12	7		
Mendenhall										
.	2		1		1	3	2	2	1	7
+	2		2		10	4	7	2	3	
++			1		1	2	2	4	2	
+++						1	1	1	1	
Total	4		4		12	10	12	10	7	7

Table 115. Organic matter, carbon and nitrogen accumulation in forest floor and mineral soil
Data from Crocker & Major (Fig. 12, 14, 15), and Crocker & Dickson (Figs. 9, 15).

Surface age (years)	70 lb./acre	Kg/m ²	90 lb./acre	Kg/m ²	180 lb./acre
Glacier Bay (18 in. profile incl. forest floor)					
Organic litter residues above surface (L, F, H)					
Organic carbon	5.1	6.5	57,850	9.5	84,550
Nitrogen	2.8	3.75	33,375	5.2	46,280
C/N Ratio	0.25	0.28	2,492	0.195	1,736
	11.2	13.4		26.7	
Mendenhall (24 in profile* incl. forest floor)					
Organic litter residues above surface (L, F, H)					
Organic carbon	9.0	12.2	108,580	16.5	146,850
Nitrogen	5.3	6.9	61,410	9.6	85,440
C/N Ratio	0.26	0.29	2,581	0.36	3,204
	20.4	23.8		26.7	

*It is unfortunate that the depth of the profiles at Glacier Bay (18 in.) is not the same as at Mendenhall (24 in), but it is thought that the comparisons are not seriously invalidated by the difference.

Table 116. Species of fungi with restricted distribution. Reported only from:

Localities	
Glacier Bay	Eagle
<u>Aspergillus ustus</u>	<u>Penicillium piceum</u>
<u>Fusarium roseum</u>	<u>Scopulariopsis sp.</u>
<u>Geotrichum candidum</u>	
<u>Penicillium aculeatum</u>	
<u>Penicillium chrysogenum</u>	
<u>Penicillium implicatum</u>	
<u>Penicillium lanosum</u>	
<u>Penicillium urticae</u>	
<u>Sporotrichum sp.</u>	
Mendenhall	
<u>Coniothyrium fuchellii</u>	
<u>Mucor anagallisporus</u>	
<u>Mucor fragilis</u>	
<u>Mucor mucedo</u>	
<u>Oospora lupini</u>	
<u>Paecilomyces varioti</u>	
<u>Penicillium herquei</u>	
<u>Penicillium purpureogenum</u>	
<u>Penicillium variabile</u>	
<u>Penicillium velutinum</u>	
<u>Phialophora fastigiata</u>	
<u>Pythium sp.</u>	
<u>Sepedonium sp.</u>	
Soil Layers	
Mineral	
Organic	
<u>Mucor fragilis</u>	<u>Phialophora jeanselaei</u>
<u>Mucor mucedo</u>	<u>Oospora lupini</u>
<u>Penicillium piceum</u>	<u>Paecilomyces varioti</u>
<u>Phialophora fastigiata</u>	<u>Penicillium aculeatum</u>
<u>Pythium sp.</u>	<u>Penicillium chrysogenum</u>
<u>Scopulariopsis sp.</u>	<u>Penicillium fellutanum</u>
	<u>Penicillium implicatum</u>
	<u>Penicillium lanosum</u>
	<u>Penicillium ochro-chloron</u>
	<u>Penicillium purpureogenum</u>
	<u>Penicillium velutinum</u>
	<u>Penicillium urticae</u>
	<u>Sepedonium sp.</u>
	<u>Sporotrichum sp.</u>

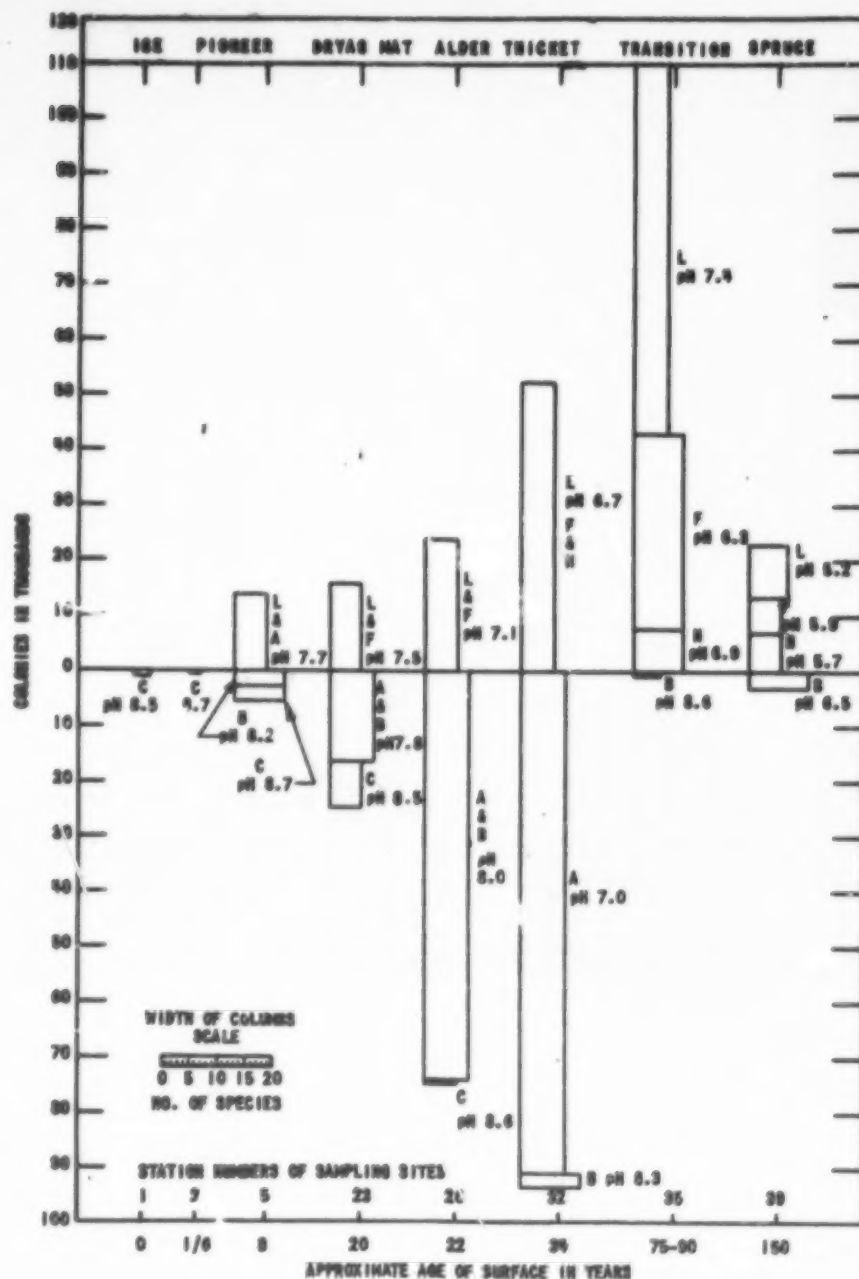


FIGURE 62

Glacier Bay, Alaska. Average numbers of colonies of fungi per gram dry weight of sample developed on five agar plates, and corrected to a dilution of 1:100. Zero line separates mineral soil (below) from overlying organic layers which have accumulated largely through deposition of plant parts. Collection stations arranged from left to right in order of increasing surface age following recession of glacier ice.

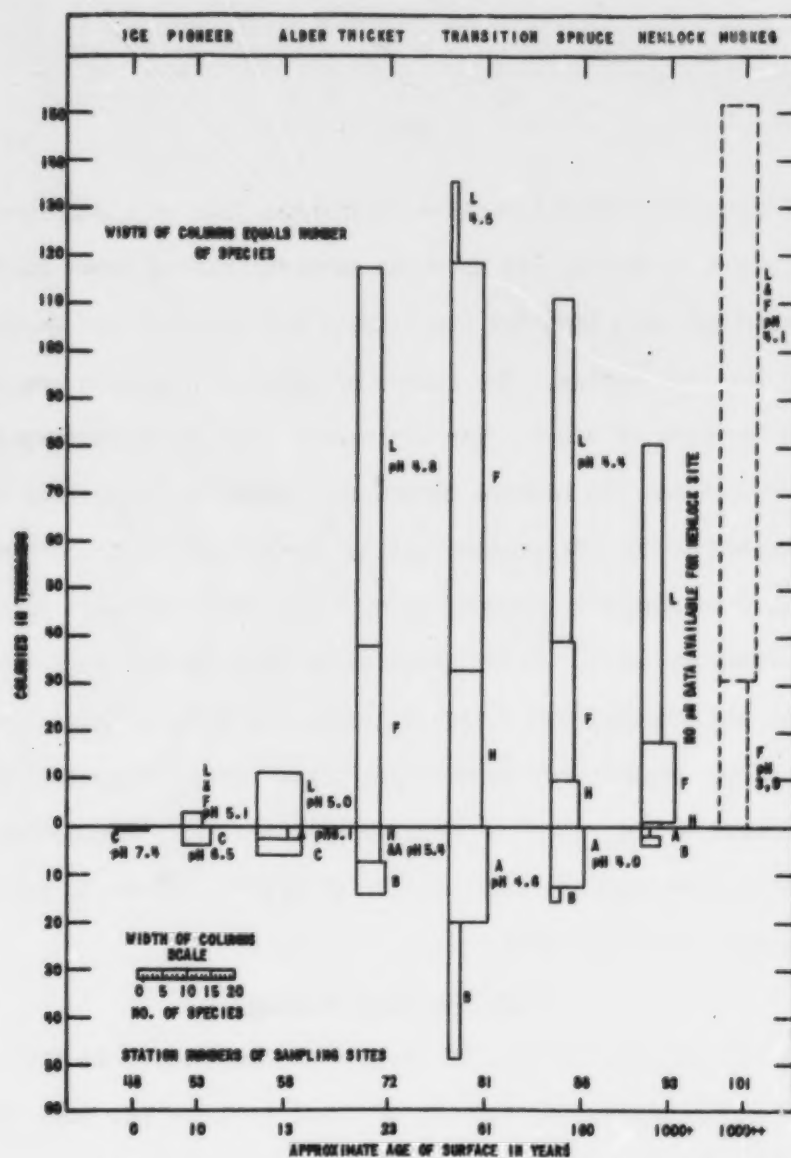


FIGURE 63

Mendenhall Glacier, Alaska. Average numbers of colonies of fungi per gram dry weight of sample developed on five agar plates, and corrected to a dilution of 1:100. Zero line separates mineral soil (below) from overlying organic layers which have accumulated largely through deposition of plant parts. Collection stations arranged from left to right in order of increasing surface age following recession of glacier ice. pH values after Crocker and Dickson (1957) approximate. Muskeg column indicated in broken lines, at right, is from nearby Eagle Glacier; the values there are more approximate because dry weight of samples was not known.

sample, based on averages of five replicates from each sample corrected to a dilution of 1:100, are shown as vertical blocks above and below the horizontal zero line for the Glacier Bay (Fig.59) and Mendenhall Glacier (Fig.60) series. The number of species from each sample is indicated by width of block. The horizontal zero lines represent the interface between the mineral parent soil material below, and the organic layers above. As pointed out by Crocker and Major, the mineral material and organic increment are kept virtually distinct in this region by the stone pavement. Elimination or at least marked reduction of freezing and thawing, and dearth of burrowing animals largely prevent mixing of the organic and mineral portions. Downward penetration by larger roots and migration of organic colloids with percolation of rain water of course provide some transfer of organic matter to parent material beneath the pebble pavement.

Sampling Restrictions

As already pointed out, the height of the columns in Figs.62 and 63 represents the number of colonies of fungi isolated per gram dry weight of sample. Had the samples been collected volumetrically from standard horizontal surface areas, the results of all the samples could be quantitatively more comparable. It might be still more satisfactory to express results per volume unit of the organic fraction of each sample on a dry weight basis. The present data are open to the criticism that, since the sizes of the organic and mineral samples are based on dry weight units, they are differently influenced by their apparent specific gravities (bulk densities), but since the organic and mineral samples are graphically

separated by the horizontal zero lines in Figs. 62 and 63, perhaps the difference is not too objectionable. The apparent specific gravity difference operates to exaggerate strongly the height of the column for the organic samples above the zero line.

Habitat Factors

Acidity of the series of samples at Glacier Bay, as ascertained with glass electrodes in a Beckman pH meter at Cincinnati at the time of preparation of dilutions, is given in Fig. 62. The data follow approximately those given by Crocker and Major which indicate that pH values decrease rather rapidly especially beneath alders as calcium carbonate is removed with the passage of time, and that pH values are higher at deeper levels and remain above neutral for over 180 years at and below the 1 foot depth in the mineral soil. The pH data of our Mendenhall samples were unfortunately lost, but data from a similar series published by Crocker and Dickson showed that the fresh till there was less alkaline; the values (Fig. 63) begin at about 7.4 and drop to about 5.0 within the first 50 years, and gradually to 4 in the following centuries.

One may see in the histograms of Figs. 62 and 63 that as the sampled areas increased in age and the organic layers accumulated, the number of fungus colonies increased through the Alnus thicket stage and into the transition forest as the Alnus died out and its stems and roots were added to the forest floor substance, decreasing as the Picea forest assumed dominance. In the Glacier Bay series (Fig. 62) the largest number of colonies occurred in the late Alnus thicket stage where the numbers were rather evenly distributed below and above the mineral surface. In

the transition stage immediately following, the number in the forest floor continued to rise while that in the mineral substratum fell sharply. These trends in mold populations follow in a notable way the nitrogen content demonstrated by Crocker and Major which reached a peak of 2490 lb./acre ($(0.28\text{kg/m}^2)/18\text{ in. profile}$) at Glacier Bay in the transition stage. Later, in the Picea forest stage, nitrogen content of the soil decreased, presumably by moving rapidly into the Picea trees. Little of the nitrogen is thought to be lost to run-off or to ground water.

In number of colonies the Mendenhall series (Fig.63) differs in three notable ways from the Glacier Bay series. The Mendenhall histogram shows (1) smaller numbers of colonies in the mineral substratum, which is an observation parallel to that of many other workers who have noted decreasing numbers of fungi downward in a profile, (2) a larger number of fungal colonies in the forest floor layers, and (3) a much slower decline in numbers following the peak in the transition community. These two latter trends follow closely the curve of nitrogen content demonstrated by Crocker and Dickson (p. 178), which rises during the first 90 years at about the same rate as at Glacier Bay, but continues to rise thereafter.

In Table 115a comparison is made of available data derived from Crocker and Major, and from Crocker and Dickson, to show differences in amounts of organic litter residues (L, F, H), organic carbon and nitrogen, and in carbon-nitrogen ratio, with increasing age of surface and also between the Glacier Bay and Mendenhall areas. These values do not represent

a measure of the total accumulation of organic matter, carbon and nitrogen in the whole ecosystem, of course, but only that portion in the soil including the forest floor; the living animals and plants, except for some surface roots, are excluded. It may be seen in the Table that although the nitrogen content is closely comparable and rises at about the same rate through the first 90 years in both areas, it has already begun to fall off at Glacier Bay by 180 years. At Mendenhall on the other hand, the nitrogen level continues to rise for over 1000 years to the value found at the latest station sampled by Crocker, our Station 93, on late-Glacial till. However, by this late Tsuga forest stage the soil is so waterlogged that much of the nitrogen is very likely unavailable to aerobic organisms.

The situation with regard to the rates of accumulation of organic residues and organic carbon is different from that of nitrogen. The values are almost twice as high at Mendenhall for comparable ages of surface as at Glacier Bay and it is not until 180 years have passed that carbon-nitrogen ratios become equal. The Crocker and Major data show a mean annual rate of net accumulation (gains less losses) to this part of the ecosystem (soil plus forest floor) for the first 70 years at Glacier Bay of about 356 lb of organic carbon and 32 lb of nitrogen per acre. Lawrence has found at Glacier Bay that the addition from leaf fall alone from an Alnus thicket 5 ft tall and 5 years old was approximately 140 lb in a single year and it is likely that the rate of nitrogen addition to the soil increases greatly as the Alnus shrubs mature, and probably continues at a high level as they die off and their woody parts

are added to the forest floor substance.

There is thus a wealth of nitrogenous organic material in the upper layers of soil available to fungi in the Alnus stage and in the following Populus transition stage in which the nitrogen previously fixed symbiotically in Alnus is absorbed by the Populus. Preliminary tests have shown that the nitrogen content of the Populus leaves suddenly rises to a level as concentrated as in the leaves of adjacent Alnus when Populus previously isolated became surrounded by Alnus thicket. Thus, the Alnus nitrogen circulates through the Populus trees of the ecosystem during the single generation of their survival, and the leaf-decomposing fungi are an important part of the circulatory mechanism during the first century following recession of the ice.

Some comment is needed here concerning the cultures from the Eagle Glacier Sphagnum-Carex muskeg, and the underlying acid peat of pH 3.9-4.1, which has been accumulating for several thousand years. It is unfortunate that the colony counts are not strictly comparable with the others because dry weights of portions of the samples are lacking. It seems that the abundance of mold fungi here is much less than at the other stations sampled and that the low pH and saturation with water may well be major influences. It must be pointed out also that although precautions were taken to avoid surface material contamination of the deep samples 104 and 105 collected with the Hiller auger, it is not possible to be certain that the organisms cultured had actually been living at those subsurface levels. But it has been tentatively assumed that the organisms identified in the culture plates were indeed living microaerobically at those depths in the peat. It is interesting to note in this connection

that Conway (1949, p. 201) had similarly isolated, from peat samples collected 10-20 cm. below the surface in Minnesota, four microaerophilic molds: Trichoderma viride, Mucor silvaticus, Penicillium sp. and Zygorhynchus moelleri.

Soil mold ecology

The medium used for isolating these fungi has glucose as a sole readily available carbon source. Thus, the principal fungi obtained are the so-called sugar fungi with those additional species of cellulose-decomposing fungi and other fungi able to utilize glucose and compounds which readily break down to glucose. In stating that the glucose present is the sole carbon source, it is assumed that any carbon available in the agar would not be used until the glucose supply had been exhausted. The fungi isolated in the present study exist in the soil, probably along with others which did not appear in the cultures, as mycelium which is actively developing, as matured spores capable of germinating at once, or as resting spores or cells such as chlamydo-spores. A more complete account of the populations under consideration would have utilized the techniques developed by Warcup (1951, 1955) to learn the nature of the fungus populations, whether they were active or only present as resting cells or spores. Further completeness would have been obtained could the suggestions subsequently published by Garrett (1956) have been followed. These include the use of direct observation, direct plating, and plating on differential rather than on single media.

In addition to the saprobic sugar fungi, there are many other nutritional groups of fungi present in the soil, and no attempt was made to isolate the species which compose them. Some of these were identified

from the fruiting material collected by Sprague in 1952. Among them are those capable of attacking cellulose, lignin, or complex proteins of animal and plant tissues including those of the symbiotic nitrogen fixers such as legumes, Alnus, Shepherdia and Dryas. The fungus biota includes also predatory fungi, aquatic Hyphomycetes, aquatic fungi inhabiting moister aerobic phases of the soil, and the larger fungi, including the Discomycetes whose mycelia penetrate the soil as well as the litter, the Hymenomycetes whose fruiting bodies were seen in many of the plots with or without the 'forest floor' layers and which in many other regions have been reported to form mycorrhiza with adjacent vascular plants as well as to make use of dead vegetable matter as food sources, and finally the Gasteromycetes which produce fruit bodies either underground or above ground. Since no attempt has been made in the present study to isolate these more complex groups of fungi, the picture of the fungi in the areas which were sampled is most incomplete.

According to Garrett's concept (1956) of the role of a fungus in the soil, the developmental phase may grow rapidly at the expense of a suitable fragment of litter. When the available nutrients in this fragment are exhausted, the fungus will fruit, producing a large number of spores and these spores will become a resting stage. They may be blown or washed to another location or they may remain where they were produced. The parent mycelium may itself become a resting body, or it may have produced one or more large thick-walled resting cells or clusters of small thick-walled cells in a sclerotium or a sclerotium-like mass. As new pieces of litter become available for colonizing, the resting cells again

become active, reproduce the developmental phase, and finally end in spore production.

The soil is permeated with a complex of filaments of these fungi. many of them may be destroyed by the violent shock of sampling so that in many cases only the spores remain; others were probably destroyed by the anaerobic and temperature conditions to which they were exposed in shipment, or in later culture (20-25°C instead of about 10°C as in nature).

The relatively large numbers of colonies found in the lower levels sampled, especially in the Glacier Bay area, indicate that adequate substrata were present for growth and reproduction, that fungus spores from upper soil layers had washed downward in the soil as shown elsewhere by Burges (1951), or that certain fungi have a greater tolerance for reduced oxygen or higher carbon dioxide concentrations. Since sampling did not reach deep levels in the parent mineral material at which no organic materials were available to support saprobic growth, the numbers of fungi at such levels are not known but may be presumed to be low.

The fungi found in the fresh till from the surface of the ice demonstrate that transport by wind is probably the method by which most fungi migrated to the areas under consideration. This is in contrast to methods of dispersal of other fungi including the aquatic and those which are carried by rodents following the ingestion of spores during the eating of such fleshy fungi as agarics or true and false truffles. Of the fungi listed in Tables 109-113, most species have air-borne spores. As additional species were added to the population, their ecological niches were found and they became part of the population or, with no adequate

substratum for their development, they died.

We have seen earlier that the mineral surface of the sampled areas consists of a pavement of stones which becomes increasingly insulated from frost and freezing action by litter and other organic materials as vegetation becomes more complex. Almost at the start of litter accumulation fractions of the dead tissues forming the litter are left in an undecomposed state or become modified to form humic acids or protein complexes. Such materials gradually decompose and may be leached downward even into the parent materials (Wakeman, 1938). Some of the lignin complexes present in the litter, fermentation and humus layers may decompose only slowly, and certain mushroom-type fungi may be among the few organisms capable of degrading them.

Unidentified cultures of Moniliaceae are abundant throughout the isolations. In the samples from which they were isolated, 50-75% of the colonies were of this type. This is a comparatively high number of colonies but conditions have not yet been developed in our Cincinnati laboratories for the satisfactory fruiting of these fungi in culture, and without fruiting, identification is usually impossible. In many cases, adequate monographic treatments for genera are not available and colonies isolated in genera such as Cephalosporium are not readily named. Since the species of the genus Phoma and its relatives are named and identified on the basis of host relations, it is not possible at the present time to name isolates from the soil, water, and other habitats not associated with identifiable living tissues of vascular plant species.

From the combined list (Table 109) there is little indication that one area or one horizon has a different mycobiota than another area or

horizon. However, when this list is broken down into its component parts
110,111,112,116) 113
(Tables / and analyzed as in Table /, it is noted that certain
habitat preferences may occur. For instance, at Glacier Bay, Mucor sp.
and Penicillium thomii occur in all stages from Dryas ~~mat~~ through Picea
sitchensis. The Mucor increases in abundance through the Populus stage
while the Penicillium decreases gradually through the same sequence. It
would be of interest to know which substrates in these habitats are
utilized by such fungi, both from the point of view of their occurrence
and of the degradational activities for which they may be responsible.
In both the Glacier Bay and Mendenhall sequences, the fact that soil
mold fungi increase through the deciduous phases of the vascular plant
succession and then decline with the increasing importance of conifer
trees, may be a reflection of unfavorable competition of litter- and
humus-decomposing higher fungi of Hymenomycetes whose mycelium, partly
in mycorrhizal association with forest trees, was in its metabolic phase
of development rather than in its fruiting phase at the time of sampling.
Such competition may have more effect on such fungi than pH factors since
the compounds present in coniferous litter and humus may be more readily
available to mushroom-type fungi than to mold-type fungi.

Summary and Conclusions

A soil plate dilution technique was used to isolate soil fungi from
two series of soil samples obtained from glacier ice and glaciated sur-
faces of various ages in southeastern Alaska.

As the natural vascular plant communities mature and as the nitrogen
content of the soil increases the populations of mold fungi increase in

numbers of species and individuals. As the litter materials increase, the nitrogen fraction is used by a larger number of organisms in the presence of greater carbon supplies in the litter. As more coniferous litter becomes available, materials which decompose slowly or poorly, if at all, are added to the forest floor, and organisms such as the mushroom-type fungi, which may be able to use these newer chemicals, such as humic acids and numerous types of lignin-containing compounds, become competitive. Under these conditions, the numbers of colonies of soil mold fungi decrease and sometimes may drop out while others may be added to the populations.

As the vascular-plant vegetation matures, more and more soil mold fungi are found in deeper layers of mineral soil.

The fermentation (F) and humus (H) layers of the forest floor, and the upper layers of mineral soil (A, B) appear to be most densely populated with fungus colonies. The large number of species in the litter layer is related to the primary decomposition processes in the litter, although the age of the samples and the technique used were not wholly adequate to determine which of the isolated fungi may be included in a group of species capable of making the initial colonization through breakdown of the cutinized layers of leaves and stems.

The soil mold populations in the two areas studied are fairly homogeneous. With the techniques used, no special trend is shown for dominance of a particular horizon by a special fungus. Certain species appeared only in pioneer communities, others in intermediate stages, and still others in mature conifer forests (Tables 110, 111). Some species were found only at Glacier Bay, others in the Mendenhall Glacier area, some in

materials on the forest floor, and others in the mineral soil (Table 116).

Because of the nature of the life cycle of the soil mold fungi and their still poorly known distribution in the soil, it is not safe, on the basis of a limited number of isolations from a limited number of samples, to assume that all species of fungi/identifiable with the technique used appeared on the isolation plates.

C. Soil fungi in miscellaneous samples

1. Lassen Volcanic National Park

Fungi are present to greater or less extent in all soils. They form a part of the biota and are important in returning minerals to the soil from dead plant and animal remains so that these substances can be used again by current vegetation. They are important in the rhizosphere and as mycorrhizal formers in the total ecosystem of any area on the earth's surface. Partly to discover what soil fungi might be present in soil samples from areas relatively undisturbed by man's activities, and partly to see how some of man's recreational activities in "wilderness" areas might affect the soil fungus populations, samples of soils were obtained on three visits to Lassen Volcanic National Park. The samples were collected so that they were not contaminated by handling, but they were not refrigerated during transport back to the laboratory. In laboratory processing, some of the samples were handled only qualitatively, a few were handled quantitatively although no replication was made and the numbers used can be referred only to the total plate counts.

The first set of samples was collected in the summer of 1956. At this time one of the sulfatara fumaroles on the rim of the crater of

Table 117. Fungi observed in isolations from retreating glacier soils, Alaska

Species	Observation Numbers			
	Total	Mendenhall Glacier	Glacier Bay Glacier	Eagle Glacier Muskeg
Moniliaceae	160	85	67	8
<u>Cephalosporium</u> spp.	53	25	26	2
<u>Cladosporium cladosporioides</u>	45	23	22	
<u>Penicillium lilacinum</u>	35	26	9	
<u>Trichoderma viride</u>	31	21	10	
<u>Mucor hiemalis</u>	28	20	8	
<u>Penicillium</u> spp.	26	10	8	8
<u>Mucor hiemalis</u> Sect. n.sp.	25	10	15	
White yeasts	23	16	6	1
<u>Phoma</u> spp.	22	3	19	
<u>Aspergillus versicolor</u>	21	12	8	1
<u>Penicillium oxalicum</u>	19	6	13	
<u>Aspergillus sydowii</u>	17	16	1	
<u>Penicillium thomii</u>	17	1	16	
<u>Cephalosporium coremioides</u>	15	6	9	
<u>Alternaria tenuis</u>	13	3	6	4
<u>Torulopsis inconspicua</u>	11	9	2	
<u>Cryptococcus albidus</u>	11	5	6	
<u>Aspergillus flavus</u>	10	10		
Dematiaceae	10	1	8	1
<u>Penicillium variabile</u>	10	10		
<u>Rhodotorula</u> spp.	10	7	3	
<u>Penicillium kapuscinskii</u>	9	8	1	
<u>Penicillium ochrochloron</u>	8	6	1	1
<u>Mucor anguliporus</u>	5	4		1
<u>Phialophora</u> spp.	5	5		
<u>Aureobasidium pullulans</u>	4	2	2	
<u>Penicillium berguiei</u>	4	2	2	
<u>Sepedonium</u> sp.	4	4		
<u>Sporotrichum</u> sp.	4		4	
<u>Aspergillus niger</u>	3	1	2	
<u>Geotrichum candidum</u>	3		3	
<u>Penicillium nr. fellutanum</u>	3	1	2	
<u>Penicillium funiculosum</u>	3	3		
<u>Penicillium lanosum</u>	3		3	
<u>Phialophora fastigiata</u>	3	1	2	
<u>Candida mesenterica</u>	2	2		
<u>Penicillium cyclopium</u>	2	2		
<u>Penicillium lividum</u>	2		2	
<u>Penicillium spinulosum</u>	2	2		
<u>Phialophora jeanselzei</u>	2	1	1	
<u>Beauveria bassiana</u>	1		1	
<u>Botrytis</u> sp.	1		1	
<u>Cladosporium herbarum</u>	1	1		
<u>Coniothyrium fuckelii</u>	1	1		
<u>Epicoecum purpurascens</u>	1	1		
<u>Fusarium oxysporum</u>	1	1		
<u>Fusarium roseum</u>	1		1	
<u>Menispora</u> sp.	1	1		
<u>Mucor</u> sp.	1	1		
<u>Mucor fragilis</u>	1	1		
<u>Mucor mucedo</u>	1	1		
<u>Oospora lupuli</u>	1	1		
<u>Paecilomyces varioti</u>	1	1		
<u>Penicillium carmine-violaceum</u>	1	1		

Table 117. . p. 2

<u>Penicillium chrysogenum</u>	1			
<u>Penicillium martensii</u>	1		1	
<u>Penicillium nigrum</u>	1	1		
<u>Penicillium purpurescens</u>	1			
<u>Penicillium rubrum</u>	1	1		1
<u>Penicillium urticae</u>	1		1	
<u>Penicillium velutinum</u>	1		1	
<u>Piptoccephalis lemmingiana</u>	1	1		
<u>Pythium sp.</u>	1	1		
<u>Scopulariopsis brevicaulis</u>	1	1		
<u>Sphaeromonas spinella</u>	1			
<u>Torulaensis fumata</u>	1	1		1
<u>Trichosporum sp.</u>	1	1		
	1	1		

Lassen Peak was still active enough that it continually emitted steam and paper put in it would be charred. This was used as a garbage incinerator. In addition to sampling soil-like materials here, a fumarole not so used was sampled and a soil sample was obtained from the base of a rock near the top of the summit trail on the rim of the crater. Other samples included soil under trees near timberline along the Summit Trail, and soils from a meadow at Cold Boiling Lake in areas visited infrequently.

The second set of samples was collected Aug. 18, 1957, and was held in a refrigerator for a considerable period of time before processing. The samples were obtained from Emerald Lake and Lake Helen and included lake water, lake bottom sediment, lake shore water-soil interface, wet soil and dry soil at progressively greater distances from the water. Lake Helen does not support fish although Emerald Lake does. Fishing is not permitted so that there is no intensive use of the lake shore except for a walking trail along the parts of it closest to the road nearby.

The third set of samples was obtained from two of the camp sites in the new Manzanita Lake Public Camp Ground a year after its inception, and from areas of heavier visitor usage at the visitor's center. This set of samples was handled more carefully and processed more quickly upon return to the laboratory since an idea of the relation to population pressure and soil fungus population was desired.

The fungal populations found in these sets of samples are given in Tables 118-122. Information about sampling sites is included.

From a series of samples of which only one represents each location sampled, it is not possible to derive conclusive information about the population of an undisturbed or a disturbed area. However, by comparison,

Table 118. Fungi observed in isolations from miscellaneous soil samples*

Species	Observation Number
<u>Penicillium</u> spp.	267
<u>Moniliaceae</u>	226
<u>Penicillium janthinellum</u>	89
<u>Mucor</u> spp.	78
<u>Trichoderma viride</u>	74
<u>Aspergillus</u> spp.	53
<u>Aspergillus niger</u>	42
<u>Penicillium lilacinum</u>	41
<u>Aureobasidium pullulans</u>	28
White yeasts	28
<u>Rhizopus</u> spp.	22
<u>Phialophora jeanselmei</u>	21
<u>Rhodotorula</u> spp.	21
<u>Cladosporium cladosporioides</u>	18
<u>Aspergillus flavus</u>	17
<u>Phoma</u> spp.	17
<u>Penicillium nigricans</u>	18
<u>Leptographium</u> spp.	15
<u>Aspergillus sydowii</u>	14
<u>Alternaria tenuis</u>	12
<u>Fusarium oxysporum</u>	12
<u>Mucor angulisporus</u>	12
<u>Cephalosporium</u> spp.	12
<u>Absidia spinosa</u>	10
<u>Penicillium thomii</u>	7
<u>Aspergillus fumigatus</u>	6
<u>Aspergillus versicolor</u>	6
<u>Sotryotrichum piluliferum</u>	6
<u>Curvularia lunata</u>	6
<u>Fusarium</u> sp.	6
<u>Gilmanella humicola</u>	6
<u>Mucor fragilis</u>	6
<u>Penicillium chrysogenum</u>	6
<u>Phialocephala dimorphospora</u>	6
<u>Stemphylium</u> sp.	6
<u>Acrospira</u> sp.	5
<u>Aspergillus ochraceus</u>	5
<u>Cladosporium</u> sp.	4
<u>Dematiaceae</u> spp.	4
<u>Epicoccum purpurascens</u>	4
<u>Paecilomyces varioti</u>	4
<u>Stachybotrys atra</u>	4
<u>Curvularia geniculata</u>	3
<u>Mucor alternans</u>	3
<u>Periconia</u> sp.	3
<u>Absidia californica</u>	2
<u>Aspergillus ventii</u>	2
<u>Geotrichum candidum</u>	2
<u>Gliocladium</u> spp.	2
<u>Gliocladium deliquescens</u>	2
<u>Gliocladium fimbriatum</u>	2
<u>Mucor racemosus</u>	2
<u>Pachybasium</u> sp.	2
<u>Paecilomyces</u> sp.	2
<u>Penicillium herquei</u>	2
<u>Penicillium ochrochloron</u>	2
<u>Scopulariopsis icelandensis</u>	2

The following species were observed only once in this set of samples:

Aspergillus nidulans, A. tamarii, A. terreus, Candida sp., C. pseudotropicalis,
Chaetomella sp., Cladosporium herbarum, Cryptococcus diffluens, Fusarium dimarum,
F. roseum, Gladiolus roseum, Gliomastix convoluta, Hemicola grisea, Mononiella
echinata, Monilia sitophila, Mortierella sp., M. isabellina, Penicillium canescens,
P. clavigerum, P. commune, P. corylophilum, P. crustosum, P. kapuscinskii,
P. martenii, P. waksmanii, Pyrenochaeta sp., P. terrestre, Pyrenomyces,
Rhizopus arrhizus, Scopulariopsis brevicaulis, Stemphylium botryosum, S. consortiale,
Thysanophora sp., Verticillium sp.

* Sources of soil samples are:

Mount Shasta, Siskiyou Co., Calif., flash fire chaparral study
 Lassen Volcanic National Park, Shasta Co., Calif., camp ground samples
 Mount Shasta, Calif., miscellaneous soil samples.
 Soil samples residues from Bacteriological study of wild soils.
 Soil samples from Mycological Society Foray, Oregon.
 Soils from Great Salt Desert, Utah.
 Lassen Park soils collected in 1957.

Table 119. Lassen Peak and Cold Boiling Lake Soil Fungi.

Sample Location	Fungi obtained*				
	<u>Trichoderma</u> <u>viride</u>	<u>Aspergillus</u>	<u>Cladosporium</u> sp.	<u>Penicillium</u> sp.	Sp.
Undisturbed fumarole, mineralized surface	3,100		100	10,000	100
Undisturbed fumarole, sand beneath mineral deposit	4,900		1,700	10,000	300
Fumarole used as garbage incinerator	15,000	niger 17,000 fumigatus 10,000	40,000	15,000	
Moist soil in shadow of rock at crater rim top of Summit Trail	13,000	niger 100			1,000
Moist soil under <u>Pinus</u> <u>albicaulis</u> at about 9500 ft	75,000				53,000
Moist soil under <u>Tsuga</u> <u>mertensiana</u> , about 9000 ft	100,000		1,000		14,000

All samples collected in open Abies magnifica forest, sedge meadow and shores of Cold Boiling Lake yielded Trichoderma viride and unidentified soil fungi in varying amounts.

* Numbers given are for comparison only and do not necessarily represent a true picture of the total population of fungi present at any one point.

Table 120. Emerald Lake and Lake Helen Fungi

Sources and Numbers of Species

Sample	Location	Habitat	Number of Species
12	Lake Helen	Water	5
13		Bottom sediment 10" in lake	5
14		Shore sediment (at water line)	6
15		Wet soil, 10' from shore	5
16		Dry soil, 100' from shore	3
17	Emerald Lake	Water	4
18		Bottom sediment 10" in lake	2
19		Shore sediment (at water line)	2
20		Shore sediment with iron oxide	6
21		Damp soil 10' from shore	2

Distribution of Species

Fungal Species	Sample Number									
	12	13	14	15	16	17	18	19	20	21
<u>Aspergillus sydowii</u>	*	*	*	*					*	
<u>Rhodotorula</u> sp.	*					*			*	
<u>Mucor</u> sp.	*		*							
<u>Phialophora</u> sp.	*		*							
<u>Cladosporium cladosporioides</u>	*									
<u>Penicillium</u> spp.		*	*	*	*	*	*	*	*	*
Unidentified spp.		*	*	*	*		*	*	*	*
<u>Penicillium janthinellum</u>		*	*	*	*	*				
<u>Penicillium chrysogenum</u>		*		*						
<u>Candida</u> sp.						*				
<u>Aureobasidium pullulans</u>									*	
<u>Phoma</u> sp.									*	

Table 121. Manzanita Lake Public Camp and Visitor Center
Soil Fungi.

Sources of Samples and Numbers of Species and Colonies

Sample	Source	Numbers of		pH
		Species	Colonies*	
1	Visitor Center, Garbage Can	26	1,000	5.5
2	Indian Lecture Bench (early)	16	250	5.7
3	Lawn at Ranger's home	14	450	6.1
4	Camp Ground, Chaparral, near Table	18	565	5.6
5	near Stove	20	550	6.5
6	Tent area	18	750	6.3
7	Jeffrey Pine, near Table	17	1,000	6.1
8	near Stove	13	1,250	7.4
9	Tent area	16	750	7.3
10	Undisturbed area under pine and chaparral	14	600	7.0

* Colonies per gram dry weight of sample

Note: Valid conclusions cannot be drawn from one set of samples with results obtained without duplication of samples or replication of platings.

Table 122. Distribution of Species.

Species	Habitat (See Table 121)									
	1	2	3	4	5	6	7	8	9	10
<u>Penicillium</u> spp.	*	*	*	*	*	*	*	*	*	*
Unidentified yeasts	*	*	*	*	*	*	*	*	*	*
Unidentified molds	*	*	*	*	*	*	*	*	*	*
<u>Trichoderma viride</u>	*	*	*	*	*	*	*	*	*	*
<u>Alternaria tenuis</u>	*	*	*	*	*	*	*	*	*	*
<u>Cladosporium cladosporioides</u>	*	*	*	*	*	*	*	*	*	*
<u>Macor</u> spp.	*	*	*	*	*	*	*	*	*	*
<u>Botryotrichum</u> sp.	*	*	*	*	*	*	*	*	*	*
<u>Penicillium nigricans</u>	*	*	*	*	*	*	*	*	*	*
<u>Epicoccum purpurascens</u>	*	*	*	*	*	*	*	*	*	*
<u>Fusarium oxysporum</u>	*	*	*	*	*	*	*	*	*	*
<u>Phialophora jeanselmei</u>	*	*	*	*	*	*	*	*	*	*
<u>Monodictys levis</u>	*	*	*	*	*	*	*	*	*	*
<u>Phoma</u> spp.	*	*	*	*	*	*	*	*	*	*
<u>Macor "fragilis"</u>	*	*	*	*	*	*	*	*	*	*
<u>Gilmanella humicola</u>	*	*	*	*	*	*	*	*	*	*
<u>Curvularia geniculata</u>	*	*	*	*	*	*	*	*	*	*
<u>Ulocladium</u> sp.	*	*	*	*	*	*	*	*	*	*
<u>Aspergillus sydowii</u>	*	*	*	*	*	*	*	*	*	*
<u>Candida pseudotropicalis</u>	*	*	*	*	*	*	*	*	*	*
<u>Chaetomella</u> sp.	*	*	*	*	*	*	*	*	*	*
<u>Cryptococcus diffluens</u>	*	*	*	*	*	*	*	*	*	*
<u>Pyricularium dimerum</u>	*	*	*	*	*	*	*	*	*	*
<u>Penicillium canescens</u>	*	*	*	*	*	*	*	*	*	*
<u>Penicillium wakehamii</u>	*	*	*	*	*	*	*	*	*	*
<u>Ulocladium botrye</u>	*	*	*	*	*	*	*	*	*	*
<u>Rhodotorula</u> spp.	*	*	*	*	*	*	*	*	*	*
<u>Aureobasidium pullulans</u>	*	*	*	*	*	*	*	*	*	*
<u>Penicillium chrysogenum</u>	*	*	*	*	*	*	*	*	*	*
<u>Pyrenochaeta terrestris</u>	*	*	*	*	*	*	*	*	*	*
<u>Aspergillus ochraceus</u>	*	*	*	*	*	*	*	*	*	*
<u>Gliocladium fibriatum</u>	*	*	*	*	*	*	*	*	*	*
<u>Penicillium thomii</u>	*	*	*	*	*	*	*	*	*	*
<u>Rhizopus</u> spp.	*	*	*	*	*	*	*	*	*	*
<u>Curvularia lunata</u>	*	*	*	*	*	*	*	*	*	*
<u>Paecilomyces varioti</u>	*	*	*	*	*	*	*	*	*	*
<u>Humicola grisea</u>	*	*	*	*	*	*	*	*	*	*
<u>Penicillium clavigerum</u>	*	*	*	*	*	*	*	*	*	*
<u>Aspergillus niger</u>	*	*	*	*	*	*	*	*	*	*
<u>Penicillium lilacinum</u>	*	*	*	*	*	*	*	*	*	*
<u>Aspergillus nidulans</u>	*	*	*	*	*	*	*	*	*	*
<u>Gliocladium deliquescens</u>	*	*	*	*	*	*	*	*	*	*
<u>Penicillium janthinellum</u>	*	*	*	*	*	*	*	*	*	*
<u>Penicillium wartsenii</u>	*	*	*	*	*	*	*	*	*	*
<u>Cephalosporium</u> spp.	*	*	*	*	*	*	*	*	*	*
<u>Aspergillus flavus</u>	*	*	*	*	*	*	*	*	*	*
<u>Penicillium crustosum</u>	*	*	*	*	*	*	*	*	*	*
<u>Monilia sitophila</u>	*	*	*	*	*	*	*	*	*	*
<u>Mortierella</u> sp.	*	*	*	*	*	*	*	*	*	*
<u>Cladosporium</u> sp.	*	*	*	*	*	*	*	*	*	*

certain types of information can be suggested. For instance, the incompletely burned garbage formed an excellent habitat, even at more than 10,400 feet, for certain types of fungi. Again, where organic wastes accumulate on the ground, as around a garbage pail or a camp stove, the soil fungus populations tend to increase. In the management of wilderness-type living areas it is suggested that this type of information be developed farther.

2. Mount Shasta

Occasional visits to Mount Shasta on summer vacation trips gave an opportunity to collect occasional miscellaneous soil samples from which some soil fungi were isolated. The work was very casual but pointed to the fact that wherever soils occur, soil molds can be isolated from them regardless of how poor they may appear to be in organic matter. Depending on runoff patterns such reservoirs of filamentous fungi can serve as a continual source of supply of fungal spores for streams and basins of any kind into which those streams feed. Such spores even occur to some extent in the muds along the shores of Lake Mead although these are probably brought down in sediments from along the Colorado River. These fungi are listed in Table 118.

One technique of clearing chaparral off of an area which could be put to productive use in the growing of a pine forest for lumber is to crush the brush and burn it under strict control so that the fire does not consume adjacent plantations or forests. Upon learning that this procedure was to be used on the lower slopes of Mount Shasta it was arranged to have a set of vials sent there for samples of soils from undisturbed areas, from established pine plantations, and from disturbed areas both

just before and just after the burning process.

Samples of litter, where available, and of soils from the 0.2", 2-6" and 6-12" levels were shipped to Cincinnati by parcel post and processed as soon as possible on arrival by techniques described earlier. The dilution level used for all samples was 1:1000, and the only medium used was neopeptone-dextrose-rose bengal-Aureomycin agar. Oven dry weights of the soils were obtained for determination of corrected estimated average number of fungal colonies per gram of soil. These values are given in Table 123. A list of 42 species and species groups of soil fungi was prepared and the distribution of these fungi in the habitats and soil depths sampled is given in Table 124.

As a result of the flash fire there was a decline in the number of colonies present in the top two inches of the soil on the day following the fire. The numbers of colonies obtained from lower levels was slightly higher after the fire than before. However, in general, neither the numbers of colonies nor the numbers of species was affected significantly by this type of burning where a prolonged surface burn can be expected to eliminate a large percentage of the fungal population for an unknown period of time.

3. Miscellaneous

a. Fungi from areas with high salt loads.

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Included in Table / are fungi which were isolated from various samples of soils obtained from moderately to highly saline areas such as certain sluggish western streams, salt lake shores and in the vicinity of plants where the salt content of the soil is extremely high as near the Great Salt Desert in western Utah. Data on the species which occur in such

Table 123

Numbers of fungiat various levels and in different habitats

Location	Number of replicates	Number of colonies per gram dry weight			
		Litter	0-2"	2-6"	6-12"
Undisturbed chaparral	2	585,000	190,000	200,000	160,000
Pine Plantation	2	595,000	275,000	114,000	83,500
Disturbed area before burning	4	-	325,000	119,000	53,000*
Disturbed area after burning	4	-	204,000	126,000	78,000

* One of the four samples lost.

Table 124

Distribution of fungus species in varying habitats and soil depths

Species	Depth and Habitat									
	Litter					2-6"				
	Chap.	Plan.	Chap.	Plan.	D.B.	D.A.	Chap.	Plan.	D.B.	D.A.
<i>Penicillium lanthornellum</i>	*	*	*	*	*	*	*	*	*	*
<i>Penicillium lilacinum</i>	*	*	*	*	*	*	*	*	*	*
<i>Aspergillus</i> sp.	*	*	*	*	*	*	*	*	*	*
Unidentified molds	*	*	*	*	*	*	*	*	*	*
<i>Penicillium</i> spp.	*	*	*	*	*	*	*	*	*	*
<i>Leptoglyphium</i> spp.	*	*	*	*	*	*	*	*	*	*
<i>Aspergillus niger</i>	*	*	*	*	*	*	*	*	*	*
<i>Mucor</i> spp.	*	*	*	*	*	*	*	*	*	*
<i>Trichoderma viride</i>	*	*	*	*	*	*	*	*	*	*
<i>Rhizopus</i> spp.	*	*	*	*	*	*	*	*	*	*
<i>Absidia spinosa</i>	*	*	*	*	*	*	*	*	*	*
<i>Fusarium</i> spp.	*	*	*	*	*	*	*	*	*	*
<i>Aspergillus flavus</i>	*	*	*	*	*	*	*	*	*	*
<i>Phialocephala dimorpha</i>	*	*	*	*	*	*	*	*	*	*
<i>Phialophora jeanselmei</i>	*	*	*	*	*	*	*	*	*	*
<i>Aspergillus fumigatus</i>	*	*	*	*	*	*	*	*	*	*
<i>Penicillium nigricans</i>	*	*	*	*	*	*	*	*	*	*
Unidentified white yeasts	*	*	*	*	*	*	*	*	*	*
<i>Aspergillus versicolor</i>	*	*	*	*	*	*	*	*	*	*
<i>Aspergillus oryzae</i>	*	*	*	*	*	*	*	*	*	*
<i>Aureobasidium pullulans</i>	*	*	*	*	*	*	*	*	*	*
<i>Cephalosporium</i> sp.	*	*	*	*	*	*	*	*	*	*
<i>Scopulariopsis canadensis</i>	*	*	*	*	*	*	*	*	*	*
<i>Trichomonospora</i> sp.	*	*	*	*	*	*	*	*	*	*
<i>Monodictys levie</i>	*	*	*	*	*	*	*	*	*	*
<i>Penicillium barneii</i>	*	*	*	*	*	*	*	*	*	*
<i>Scopulariopsis brevicaulis</i>	*	*	*	*	*	*	*	*	*	*
<i>Ulocladium</i> sp.	*	*	*	*	*	*	*	*	*	*
<i>Cladosporium herbarum</i>	*	*	*	*	*	*	*	*	*	*
<i>Curvularia geniculata</i>	*	*	*	*	*	*	*	*	*	*
<i>Curvularia lunata</i>	*	*	*	*	*	*	*	*	*	*
<i>Gileanella humicola</i>	*	*	*	*	*	*	*	*	*	*
<i>Paeclonovora</i> sp.	*	*	*	*	*	*	*	*	*	*

Table 124 (Contd.)

Species	Depth and Habitat													
	Litter			0-2"			2-6"			6-12"				
	Chap.	Plan.	Chap.	Plan.	D.B.	D.A.	Chap.	Plan.	D.B.	D.A.	Chap.	Plan.	D.B.	D.A.
<i>Rhodotorula</i> sp.						*								
<i>Rosellinia</i> <i>artispore</i>						*								
<i>Paecilomyces</i> <i>varioti</i>						*								
<i>Penicillium</i> <i>commune</i>							*							
<i>Gliocladium</i> <i>deliquescens</i>									*			*		
<i>Gliomastix</i> <i>convoluta</i>												*		
<i>Penicillium</i> <i>hamacineklil</i>												*		*
<i>Penicillium</i> <i>ochrochloron</i>												*		*
<i>Periconia</i> sp.												*		*

Chap. - Chaparral, undisturbed

Plan. - Pine plantation, undisturbed

D.B. - Disturbed area before burning

D.A. - Disturbed area after burning

* - Indicates presence of listed species

places are included here to emphasize the fact that large numbers and a variety of kinds of soil fungi occur in most unexpected habitats. The spores and other disseminules of such fungi are usually available for dissemination by water or wind to other areas where they may continue their growth processes at the expense of organic matter in the new environment, whether that organic matter is available for a decay process or whether it is a useful piece of fabric or equipment for man's activities.

Because soil fungi have been found in number in such habitats, it is reasonable to assume that soil fungi may also be found to greater or less extent, depending on the amount of available organic matter, in estuaries and tidal flat muds. To date, however, very little work has been done in this area although the so-called marine fungi have been isolated from inland salt waters such as the Salton Sea (Anastasiou, 1963).

b. Fungi of stream banks

Rather than develop a more elaborate presentation, the reader is referred to the chapters on Fungi from Streams (IV) and Fungi in Acid Mine Drainage (V) for information on the occurrence of soil fungi in these habitats.

XIV. Studies of Environmental Samples for Fungi

A. Introduction

It has long been recognized that fungi are ubiquitous organisms as well as being omnivorous. The study of various species and species groups in relation to their occurrence in various special habitats has been mentioned occasionally above. Here can be mentioned other species groups with narrow or broad spectra of distribution especially in relation to some of man's activities where fungi of use in some types of

deterioration such as organic matter in sewage, are nuisances in other types of deterioration such as the rotting of fabrics by so-called mildews, the occurrence of a variety of fungi on the human body where they can become nuisances in the case of wounds, the occurrence of some fungi as laboratory contaminants and as contaminants of various materials throughout man's experience, and the occurrence of fungi in the air.

In some cases some of these areas have come under intensive study in the Fungus Studies Laboratory or in associated laboratories. The occurrence of fungi in used bedding materials, while of importance as a source of fungal spores yielding a musty or otherwise unpleasant odor, or an allergenic condition, is closely related to the possible deterioration of the material with which the contaminating fungi are associated. The following study resulted from asking the question: "Has the presence of molds in used and reconditioned mattresses a public health significance?"

B. Fungi in Used Bedding Materials*

Few workers have been concerned with the occurrence of bacteria and fungi within used bedding, bedding materials, and upholstered furniture. Such organisms may be present in the raw materials, added during normal use and storage, multiply under certain conditions of humidity, and may be transferred to new materials when new and used materials are garretted.

Conant et al. (1935) studied the mold spore populations of pillows, mattresses, chairs, davenports and puffs, filled with kapok, hair, feathers,

*Reproduced with permission from Applied Microbiology 6:169-173, 1958.

cotton, and wool. Rhizopus nigricans was found 32 times; Chaetomium sp., 19; Aspergillus niger, 9; Aspergillus sp., 7; Penicillium sp., 20; Macrosporium sp., 1; and Alternaria sp., 2. Swaebly & Christensen (1952) conducted a study of molds and bacteria in house dust, furniture stuffing, and the air of homes in the St. Paul and Minneapolis area, and reported that used furniture and filling materials contained larger numbers of fungi than new. Lloyd & Foter (1955; Foter & Lloyd, 1955), examined bedding which was artificially contaminated with nonsporulating and sporulating bacteria and spores of Aspergillus niger. They reported that treatment in a 300 cu ft chamber, with dry heat and formaldehyde was no more effective than treatment with dry heat alone at 230°F for 2 1/4 hours in killing the test organisms on the surfaces of mattresses. Nonsporulating organisms were killed while bacterial and mold spores were not, and inside the mattresses little to no germicidal effect was noted. Wide temperature variation inside the test chamber was observed.

To determine the efficacy of the dry heat treatment (230°F for 2 1/4 h r) on used mattresses in the process of renovating them for reuse, a series of samples was obtained and portions of these were examined for the bacterial and fungal populations present. New and used materials before treatment, and materials subjected to various degrees of dry heat treatment were studied.

The present report is concerned with the identification of saprobic, potentially pathogenic or potentially allergenic fungi found in used bedding materials, and in the presence and extent of these fungi in untreated or heat treated bedding.

Experimental Methods.

Sampling

Representative samples were collected under aseptic conditions from various mattresses at a bedding renovation plant according to a predetermined sampling scheme and returned to Cincinnati for examination. The history of use, location of use, number of persons using them, and subsequent conditions of storage were obtained, both before and after treatment, by the use of aseptic die punches (2 in. in diam.) in the case of ticking, and grab samples in the case of felt.

To determine the numbers and types of fungi present in used mattresses, 60 mattresses were selected, and of these, 12 were sampled at random by removing a portion of ticking from the periphery and from the center of the mattress, and a portion of felt from the periphery and from the center of the mattress. Another series of mattresses which had been exposed to various dry heat treatments was sampled in the same way.

A second type of sampling was used to study the effect of revising the heat treatment, using existing equipment, to increase its efficacy without altering the physical condition of the regarnetted filler. The mattresses were disassembled and the fillings regarnetted. New material was added to make up any losses in weight. The regarnetted cotton fill was mechanically cut and rolled into batts. Samples were taken from the exterior and interior of 24 batts selected at random. Of these batts 18 were placed at different levels on the heat treatment chamber racks. Samples were collected from each batt before and after heat treatment to gain a better understanding of the efficacy of the varying temperatures within a single chamber. Thermocouple lead wires were attached to both

the exterior and interior of 6 batts which were placed in different areas in the chamber. Lead wires were also placed adjacent to the chamber thermoregulator. Initial temperature readings were recorded and subsequent readings made at 15-min. intervals.

Methods

Two techniques were used to prepare samples for plating. In preliminary studies, a known amount of the sample to be tested was washed or shaken in sterile distilled water and dilution plates were made from this in duplicate or triplicate. In the second, 1 g of the sample, with 99 ml of sterile distilled water, was blended in a Waring Blendor at high speed, and 3.3 ml portions were distributed among triplicate sets of three plates each.

Plating media for fungi were of two types: (1) rose bengal - chlortetracycline agar (described above as neopeptone-dextrose-rose bengal-Aureomycin agar) was used to obtain the largest number of fungal species present by inhibiting bacterial growth, without rapid coverage of the plates by spreading fungi, and (2) Sabouraud-actidione agar (Sabouraud agar, Difco, to which were added 35 ug/ml chlortetracycline and 0.5 mg/ml actidione) was used to inhibit the growth of saprobic fungi and encourage the growth of as many pathogenic fungi as possible (Ajello & Goetz, 1954).

All plates were incubated at room temperature and in the presence of light for 1 week, counted, species inventories recorded, and isolations made. After 1 week exposure at room temperature, the agar in the plates dried and no further changes occurred in the populations.

Results

Results of this study are presented in two ways: (1) an indication of the numbers of colonies and species of fungi present in used untreated and treated mattress materials, and (2) methods of study of fungi in such habitats.

Lists of species of fungi found in the materials tested are presented in Tables 126, 127, and 130. / These tables list only those species which make up more than 0.01 and 0.1 per cent of the total colonies isolated from the mattress materials tested. The species found less frequently are listed in appendices to each table. At the bottom of each column is presented the total number of colonies isolated from a particular set of samples (see next paragraph) on which the percentages for each species are based. Exposures made at varying temperatures within the same treatment chamber are not tabulated separately.

Sets of samples from which isolations were made have been designated 126-129 in Tables / by Roman numerals. These are identified in Table 125. It will be noted that in the different platings from the same basic set of samples (sets IV and V, and sets VI and VII) the same species were not always obtained in each series.

Mattresses selected at random for study were placed on racks in a 300 cu ft. heat treatment chamber and thermocouples were attached to the surface and internal contents of the mattresses to determine the temperatures of exposure. At each level, different surface and internal temperatures were obtained although the chamber thermostat was set to deliver an even heat throughout the chamber. The higher the mattresses were placed on the chamber racks the fewer colonies of a species developed, although levels of

Table 125. Sources of mattress materials.

Set Number	Treatment	Source
I	Unheated	Preliminary samples for determination of sampling procedure, plating techniques and population analysis, shaken.
II	Heated	
IV	Unheated	Ticking and felt sampled as outlined above but dilutions used were unusually high for adequate fungus sampling, shaken.
V	Heated	
VI	Unheated	Same as IV and V but with lower dilutions, blended.
VII	Heated	
VIII	Unheated	Batts garnetted from several felts after ticking was removed and before heat treatment, blended.
IX	Heated: outside of batt	
	Heated: inside of batt	
X		

Table 128. Numbers of species of fungi isolated from used mattress materials.

Set	No. of Samples	Species			
		Knee Length Agar		Sabouraud Agar	
		Untreated	Treated	Untreated	Treated
I, II	3	26	9	N.B.*	N.B.*
IV, V	12	43	22	5	1
VI, VII	12	41	45	22	12
VIII, X (inside)	27	25	36	19	4
IX (outside)	27	--	32	--	14
Total	54	69	66	37	23

* N.B. = Not sampled.

Table 129. Estimated total fungus colonies isolated from used mattress materials.

Set	No. of Samples	Species			
		Knee Length Agar		Sabouraud Agar	
		Untreated	Treated	Untreated	Treated
I, II	2	25,500	18,550	N.B.*	N.B.*
IV, V	12	17,468,660	550,000	500	100
VI, VII	12	48,780	21,440	7,920	1,650
VIII, X (inside)	21	15,738	2,035	2,375	60
IX (outside)	21	-----	3,670	-----	575
Total	45	17,588,678	591,695	10,795	2,385

* N.B. = Not sampled.

Table 130. Fungi observed in isolations from used bedding materials.

Species	Observation Number
<u>Basiliaceae</u>	167
<u>Glomeraria glaucosporica</u>	107
<u>Penicillium</u> spp.	98
<u>Aspergillus niger</u>	95
<u>Aspergillus</u> spp.	89
<u>Aspergillus chevalieri</u>	86
<u>Trichoderma viride</u>	80
<u>Aspergillus ustulatus</u>	76
<u>Aspergillus nidulans</u>	76
<u>Chaetomium</u> spp.	68
White yeasts	66
<u>Aspergillus pallidus</u>	66
<u>Penicillium lilacinum</u>	65
<u>Penicillium</u> spp.	63
<u>Alternaria tenuis</u>	55
<u>Chaetomium globosum</u>	45
<u>Aspergillus flavus</u>	44
<u>Aspergillus</u> spp.	34
<u>Aspergillus restrictus</u>	30
<u>Aspergillus</u> spp.	28
<u>Stachybotrys atra</u>	27
<u>Penicillium funiculosum</u>	26
<u>Aspergillus varicolor</u>	21
<u>Monodelia echinata</u>	21
<u>Penicillium chrysogenum</u>	21
<u>Monor glaucus</u>	19
<u>Aspergillus arbus</u>	17
<u>Monor</u> spp.	14
<u>Penicillium martenii</u>	14
<u>Aspergillus ochraceus</u>	12
<u>Syncephalastrum racemosum</u>	12
<u>Aspergillus fumigatus</u>	11
<u>Epilobium purpurascens</u>	10
<u>Penicillium oxalicum</u>	10
<u>Geotrichum candidum</u>	9
<u>Penicillium thomii</u>	9
<u>Aspergillus boerhaavia</u>	7
<u>Monor fragilis</u>	7
<u>Aspergillus ruber</u>	6
<u>Penicillium varioti</u>	6
<u>Penicillium velutinum</u>	6
<u>Phoma</u> sp.	6
<u>Aspergillus clavipes</u>	5
<u>Aspergillus tamarii</u>	5
<u>Aspergillus unguis</u>	5
<u>Gliocladium roseum</u>	5
<u>Penicillium purpuroscentum</u>	5
<u>Cryptococcus diffluent</u>	4
<u>Curularia lunata</u>	4
<u>Penicillium citrinum</u>	4
<u>Penicillium rubrum</u>	4
<u>Aspergillus cornuiformis</u>	3
<u>Aspergillus clavatus</u>	3
<u>Aspergillus fischeri</u>	3
<u>Aspergillus terreus</u>	3
<u>Aspergillus niger</u>	3
<u>Aspergillus</u> spp.	3

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<u>Mucor racemosus</u>	3
<u>Penicillium crustosum</u>	3
<u>Penicillium variabile</u>	3
<u>Phialophora jeanselmei</u>	3
<u>Rhodotorula glutinis</u>	3
<u>Sporotrichum sp.</u>	3
<u>Sporotrichum ?schenckii</u>	3
<u>Tetracocco sporium paxianum</u>	3
<u>Acremonium sp.</u>	2
<u>Ascomycete sp.</u>	2
<u>Candida parapsilosis</u>	2
<u>Chaetomium funiculosum</u>	2
<u>Chloridium chlamydosporum</u>	2
<u>Cladosporium herbarum</u>	2
<u>Fusarium solani</u>	2
<u>Helminthosporium sp.</u>	2
<u>Myrothecium verrucaria</u>	2
<u>Nigrospora sphaerica</u>	2
<u>Poria ambigua</u>	2
<u>Rhizopus oryzae</u>	2
<u>Rhizopus rhizopodiformis</u>	2
<u>Rhodotorula mucilaginosa</u>	2
<u>Scopulariopsis sp.</u>	2
<u>Stemphylium consortiale</u>	2

The following species were observed only once in this series of samples:

Arachniotus citrinus, Aspergillus candidus, A. nidulans, A. ustus, Botrytis cinirea, B. terrestris, Botryotrichum piluliferum, Chaetomium sp., C. brasiliense, Choanephora cucurbitarum, Coniothyrium fuckelii, Cryptococcus albidus, Curvularia sp., Doratomyces stemonitis, Endomyces sp., Fusarium aquaeductum, F. roseum, Penicillium Capsulatum, P. berqueti, P. janthinellum, P. pallidum, P. stipitatum, Pestalotia sp., Phialophora sp., P. lagerbergii, Sepedonium sp., ?Sordaria sp., Sporotrichum laxum, Stemphylium sp., S.lanuginosum, Torulopsis candida, Trichoderma alba, Trichosporon sp., Tritirachium album.

heat in the chamber had little effect on the number of species. In a sense, this disparity can be interpreted as population resistance to the heat treatment although it is more likely that heat did not penetrate the batts effectively.

Table / ¹²⁸ gives the number of species and Table / ¹²⁹ the number of colonies of fungi isolated from untreated and heat treated mattress materials on rose bengal-chlortetracycline and Sabouraud-actidione agars. Following heat treatment, sets VI-VII (Table / ¹²⁵) and VIII, IX, X yielded more species on rose bengal-chlortetracycline agar while treatment appeared to discourage species in sets I-II and IV-V. Isolations on Sabouraud-actidione agar indicate that heat treatment discouraged certain species in all sets compared. On the other hand, numbers of colonies were reduced following heat treatment by 3 to 50 per cent of the number of colonies found on untreated materials using rose bengal-chlortetracycline agar, and by 2.5 to 20 per cent the number of colonies on untreated material using Sabouraud actidione agar.

Several groups of fungi appeared on the isolation plates. The saprobic sugar fungi, represented by members of the genera Absidia, Mucor, Rhizopus and Syncephalastrum, usually occur on simple carbohydrate substrates. Among the species which are cellulolytic, and which may thus cause a breakdown of the materials composing the mattresses under proper conditions of climate and storage, are: Alternaria tenuis, Aspergillus fumigatus, Chaetomium globosum, Cladosporium cladosporioides, C. herbarum, Curvularia lunata, Fusarium oxysporum, F. solani, Myrothecium verrucaria, Stemphylium consortiale, and Trichoderma viride. Among these fungi, and

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in the lists in Tables / are species whose spores cause allergies if inhaled in large numbers. The better known of these include Alternaria tenuis, Aspergillus fumigatus, A. niger as well as other species of Aspergillus and some of Penicillium. Among those molds whose spores are common members of air populations are Alternaria tenuis, Cladosporium clado-sporioides, and Epicoccum purpurascens. The dry-spored molds are also found commonly in the air and a number of species other than those listed here have been implicated in allergies. Many of these species have been found in various types of household dusts.

It is interesting that in some of the samples large numbers of yeast colonies were found. These have not been studied to determine if they include one or more potential pathogens.

Among the group of species listed are fungi which may be associated with seeds in storage and during germination. These may represent species whose disseminules are able to survive on materials in storage and germinate when favorable conditions occur.

On Sabouraud-actidione agar, many species were isolated which fall into the groups mentioned above. Several colonies of a fungus which is morphologically identical with Sporotrichum schenckii, a potential pathogen, have been isolated from untreated and heat treated batts of felt. The untreated sample came from the surface of a batt of regarnetted felt, the sample of treated felt came from the inside of a regarnetted felt batt. It is not possible to decide whether the fibers on which the spores of this fungus were found came from a position adjacent to the ticking or from the inside of a mattress pad. Other fungi potentially pathogenic for man, producing pneumonia-like disorders upon

inhaling of spores (Conant, et al. 1954), which were isolated from the mattresses were Aspergillus fumigatus and Geotrichum candidum, the latter being a relatively normal inhabitant of the respiratory tract of man.

The small number of samples of new mattress materials which were tested indicated that fungal contaminants are present on such materials in considerable numbers. Studies of multiple control samples are necessary to judge the number of spores or kinds of fungi represented on new materials.

Discussion

In a study on the ability of mold spores to germinate at various humidities, Snow (1949) showed that species such as those which have
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been isolated in this series of tests (Tables / and/) could germinate at controlled humidities from 100% to as low as 65%. Several species of Aspergillus, including strains of species isolated from these materials, could germinate at humidities between 65 and 75%.

Since the materials under study are known to have been made of cotton, the fungi present could be assumed to be of two types: (1) those using cellulose and its degradation products as nutrients, and (2) those brought to the habitat by air currents and contact with various objects during use and storage. Should humidities become high enough, or should the mattress become damp or water soaked, certain species could be assumed to grow and reproduce in the habitat. All the species in the group which can use cellulose seem to be able to grow and reproduce in the habitat under proper environmental conditions.

In a heterogenous population of fungi such as the one encountered here, many species can germinate at relatively low humidities, utilize

the cellulose or the various impurities, and leave by-products for additional species to use. Some of these secondary invaders, as well as the primary contaminants, can be of importance to man as pathogens and allergens.

From the evidence presented by the isolation of fungi from these materials, definite need is indicated for improvement in treatment of mattress materials being reconditioned for further use as bedding components. Because of the poor germicidal efficiency of dry heat, other methods such as gaseous disinfection should be considered for this process.

Summary

Relatively large numbers of fungal species, represented by large numbers of spores, are present in mattress materials following removal of the mattress from storage, after heat treatment, and preceding renovation in which used materials are employed.

Dry heat treatment for sterilizing bedding materials being reconditioned for re-use is inadequate for the reduction of potential fungal activity. Species of fungi responsible for degradation of cellulose or able to cause allergies or pathogenicity in man have been found following dry heat treatment of such materials.

C. Fungi in the Air

A wide variety of fungal spores is found in the air. Gregory (1961) has discussed the occurrence, transport and means of deposition of fungi in and from the air in considerable detail. Ingold (1953, 1965) has shown how fungus spores reach the air. A large number of surveys have been made of communities where the sampling of the air spora has been

carried out in an effort to pinpoint the sources of allergenic species of fungi and to demonstrate the seasonal distribution of these fungi. Some of the more recent of these reports have been summarized by Adams, et al., (1961).

In the spring of 1953, Dr. M. J. Foter collected 22 sets of samples of air using a number of techniques. Of these, 19 sets of plates and flasks were collected for fungus studies in tandem with similar flasks and plates for the study of air populations. The following notes were made on the plates exposed for fungal studies using media prepared in the mycology laboratory.

In obtaining samples of air a Venturi tube sampler was used to which were attached a number of types of air samples. Dates for each Series of samples and relative data are given in Table 131. In sample series II and III only a capillary impinger (using 25 ml Koser's citrate broth as a collection fluid) was used as a collecting device. Beginning with Series IV and throughout the remainder of the series sieve plates were used with the agar being dextrose-peptone-salts-rose bengal-streptomycin (50 ppm). Beginning with Series VII the Schipe Impinger replaced the capillary impinger using the same broth. Beginning with Series VII the sieve plates contained phytone-dextrose-salts-rose bengal-aureomycin (35 ppm.) agar. Also starting with series VII, a hydrosol membrane filter was used in which the membrane filter was moist through the exposure period, and an aerosol membrane filter was used in 7 of the series in which the filter was not moistened until after the sample was collected.

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Table / lists the site and collection data for the 19 series of

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samples. In Table / are given the numbers of fungal colonies arising from

Table 131. Sampling Sites and Data.

Date	Series	Location	Rate of Sampling	Amount of air sampled
1-27	II	Glendale, Ohio, Sewage Treatment Plant	3 l/min	Test 1 & 4 - 1 cu ft; 2 & 5 - 2.5 cu ft; 3 - 5 cu ft.
1-29	III	Ditto	3 l/min	Test 1 - 1 cu ft; 2 & 3 - 2.5 cu ft.
2-5	IV	Ditto	ditto	Test 1 - 2 cu ft; 2 - 5 cu ft; 3 - 10 cu ft; 4 - 20 cu ft.
2-10	V	Ditto	ditto	Test 1 & 3 - 5 cu ft; 2 & 4 - 10 cu ft.
2-12	VI	Ditto	ditto	Test 1 & 4 - 1 cu ft; 2 & 5 - 2.5 cu ft.; 3 - 5 cu ft.
3-4	VII	Ditto	ditto	Test 1 & 3 - 5 cu ft; 2 & 4 - 10 cu ft.
3-5	VIII	Ditto	ditto	Tests 1, 2, 3, 4 - 5 cu ft.
3-10	IX	Ditto	ditto	Test 1, 3 - 10 cu ft.; 2 - 5 cu ft.
3-12	X	Ditto	ditto	Test 1, 2, 3 - 10 cu ft.; 4 - 5 cu ft.
3-17	XI	Ditto	ditto	Tests 1, 2, 3, 4 - 10 cu ft.
3-19	XII	Ditto	ditto	Tests 1, 2, 3, 4 - 10 cu ft.
3-26	XIII	Ditto	ditto	Tests 1, 2, 3, 4 - 10 cu ft.
4-2	XIV	Ditto	ditto	Tests 1, 2, 3, 4 - 10 cu ft.
4-7	XV	Ditto	ditto	Tests 1, 2, 3, 4 - 10 cu ft.
4-9	XVI	Ditto	ditto	Tests 1, 2, 3 - 10 cu ft.
4-14	XVII	Dayton, Ohio, Sewage Treatment Plant	ditto	Tests 1, 2, 3 - 10 cu ft.
4-22	XVIII	Ditto	ditto	Tests 1, 2, 3 - 10 cu ft.
5-6	XX	Alleys, Cincinnati, Ohio	ditto	Tests 1, 2, 3 - 10 cu ft.
5-20	XXI	West End area	ditto	Tests 1, 2, 3 - 10 cu ft.
6-2	XXII	Ditto	ditto	Tests 1, 3 - 10 cu ft; 2 - 15 cu ft.

Note: Series II - XVI were sampled in the vicinity of the receiving box where sewage entering the plant and sewage which has passed over the trickling filter are mixed resulting in a fine spray passing into the air. The opening of the sampler was placed at various distances above or away from this influent mixing box.

Series XVII and XVIII were sampled between a high rate and a low rate trickling filter in a field of 20 1/2 acre trickling filters, at various heights above the filters.

Series XIX-XXII were sampled in city streets at unspecified heights above the street.

Table 132. Fungi in air sampling, 1953.

Date	Series*	Tests	Total Count	Av. no. fungi		Av. no. fungi per 1 cu. ft. per 20 cu. ft., air	Percentages total count obtained by				MA Filter	AA Filter
				air	per 1 cu. ft.		Capillary Impinger	Schlige Impinger	Sieve Plate			
1-27	II	5	40	4.5		89.6	100.0	-	-	-	-	-
1-29	III	3	14	3.1		61.3	100.0	-	-	-	-	-
2-5	IV	4	227	8.1		162.8	10.0	-	-	-	-	-
2-10	V	4	254	6.0		108.7	5.1	-	-	-	-	-
2-12	VI	5	307	14.2		284.8	4.3	-	-	-	-	-
3-4	VII	4	528	19.7		394.5	-	22.0	-	-	8.8	16.1
3-5	VIII	4	226	11.3		226.0	-	7.1	-	-	17.7	22.6
3-10	IX	3	182	7.5		152.0	-	40.8	-	-	10.1*	10.6
3-12	X	4	723	24.4		512.0	-	24.4	-	-	4.4	16.4
3-17	XI	4	676	16.9		338.0	-	12.7	-	-	11.9	-
3-19	XII	4	1503	37.6		751.5	-	13.8	-	-	21.5	-
3-26	XIII	4	305	7.6		152.5	-	24.3	-	-	7.5	-
4-2	XIV	4	915	22.9		458.5	-	30.1	-	-	4.8	-
4-7	XV	4	1052	26.4		537.0	-	28.3	-	-	6.8	-
4-9	XVI	3	862	33.3		666.9	-	44.0	-	-	5.7	-
4-14	XVII	3	692	27.9		558.5	-	25.0	-	-	9.2	10.5*
4-22	XVIII	3	1737	57.9		1124.6	-	22.5	-	-	27.4	12.1
5-6	XX	3	1021	34.0		681.0	-	34.5†	-	-	-	-
5-20	XXI	3	4573	157.5		3150.6	-	91.4	-	-	1.9	-
5-27	XXII	3	389	16.3		326.0	-	12.3	-	-	41.6	9.8
				Average			6.5	28.9			12.7	14.0

* - 1 plate only

† - 2 plates

‡ - 3 plates

F - Material filtered through NA filter.

the series of samples obtained together with a percentage value for the numbers of colonies recovered using each of the techniques listed above.

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Table / includes a list of the species of fungi obtained using all techniques together with the number of times each species was observed in the whole experiment.

From the results obtained in this series of samples, it appears to me that the best general sampling device is the sieve sampler, at least for this series. Since no sedimentation plates were exposed concurrently, it cannot be determined whether the sieve sampler gives a better idea of the viable air spora than the sedimentation plate. From the results of other samplings noted in the literature, however, it would appear that data obtained with the sieve sampler would be more reliable. With increased efficiency of equipment, other types of samplers may yield different types of information of a more accurate nature than those described here.

D. Occurrence of Molds and Yeasts in Dairy Products*

In literature on dairy and food microbiology, as represented by reference and text books by Tanner (1944), Hammer (1958), and Frazier (1958) the technique for separating bacteria from yeasts and molds in the examination of dairy products relies heavily on the acidification of media. This is reflected by the protocols adopted by the 11th Edition of Standard Methods for the Examination of Dairy Products (APHA, 1960), which also specifies acidification of media in which fungi of any types are to be examined.

As early as 1944 Smith and Dawson used Rose Bengal, a vital stain,

*Reproduced with permission from Mycopathologia et Mycologia Applicata 35: 281-289, 1968.

Table 133. . Fungi observed in samples of air spora.

Species	Observation Number
<u>Moniliaceae</u>	186
<u>Cladosporium cladosporioides</u>	166
<u>Penicillium spp.</u>	150
<u>Alternaria tenuis</u>	119
<u>Aspergillus niger</u>	67
<u>Aureobasidium pullulans</u>	64
<u>White yeasts</u>	59
<u>Epicoccum purpurascens</u>	39
<u>Penicillium lilacinum</u>	38
<u>Fusarium oxysporum</u>	36
<u>Rhodotorula spp.</u>	36
<u>Phialophora jeanselmei</u>	32
<u>Aspergillus flavus</u>	25
<u>Trichoderma viride</u>	25
<u>Aspergillus versicolor</u>	22
<u>Aspergillus fumigatus</u>	21
<u>Penicillium funiculosum</u>	21
<u>Phoma spp.</u>	21
<u>Geotrichum candidum</u>	17
<u>Penicillium ochrochloron</u>	15
<u>Penicillium variabile</u>	12
<u>Cladosporium herbarum</u>	10
<u>Fusarium roseum</u>	9
<u>Stemphylium sp.</u>	8
<u>Aspergillus chevalieri</u> var. <u>intermedius</u>	7
<u>Aspergillus flavipes</u>	6
<u>Aspergillus ochraceus</u>	6
<u>Mucor spp.</u>	6
<u>Aspergillus spp.</u>	5
<u>Aspergillus candidus</u>	5
<u>Monilia sitophila</u>	5
<u>Mucor fragilis</u>	5
<u>Rhizopus spp.</u>	5
<u>Trichosporon spp.</u>	5
<u>Fusarium aquaeductuum</u>	4
<u>Penicillium martensii</u>	4
<u>Fusarium spp.</u>	3
<u>Paecilomyces varioti</u>	3
<u>Aspergillus amstelodami</u>	2
<u>Aspergillus oryzae</u>	2
<u>Aspergillus tamaris</u>	2
<u>Dendryphium sp.</u>	2
<u>Echinobotryum atrum</u>	2
<u>Paecilomyces sp.</u>	2
<u>Penicillium oxalicum</u>	2
<u>Sclerotium rolfsii</u>	2

The following species were observed only once in this series of isolations:

Acremonium sp., Aspergillus clavatus, Basidiomycete, Candida parapsilosis, Cephalosporium sp., Chaetomium sp., Gliocladium roseum, Monosporium silvaticum, Monotospora sp., Mucor plumbeus, Paecilomyces elegans, Penicillium chrysogenum, P. herquei, P. thomii, Pestalotia sp., Phoma herbarum, Pseudopeziza americana, Rhizopus arrhizus, Rhodotorula graminis, Triposporium myrtii, Verticillium sp.

for slowing down the rate of growth of fast growing molds in mixed primary isolation media used in soil population sampling. This dye also eliminated some types of bacteria. In 1950, Martin improved this medium by using penicillin and streptomycin, and in 1954 Cooke (see Chapter II above) added Aureomycin for substrates in which large numbers of bacteria could be expected.

Because a medium containing rose bengal and antibiotics has yielded large numbers of colonies and species of fungi from soils, polluted waters, etc., in the Fungus Studies Laboratory, we decided to determine the utility of such a medium in the recovery of fungi from dairy products.

Methods

Samples of cheese, butter, buttermilk, instant non-fat milk, sweetened condensed milk, whipping cream, homogenized milk, skim milk, half and half, chocolate dairy drink, and coffee cream were obtained by random over-the-counter purchase in grocery stores; samples of ice cream stabilizers and flavorings were obtained from laboratories of dairies producing ice cream, and raw milk and cream were obtained directly from dairies.

The samples were prepared and plated according to the techniques prescribed by Standard Methods (APHA, 1960), and according to the technique developed in the Fungus Studies Laboratory. Potato dextrose agar (Difco), and Cooke Rose Bengal Agar (Difco) were used in tandem, after rehydration according to the manufacturer's labeled instructions; an antibiotic was then added to the latter medium. Chlortetracycline hydrochloride (Aureomycin) was obtained from the manufacturer, Lederle Laboratories, and one

gram of the product was dissolved in 150 ml of distilled water. This was stored in the refrigerator and sterilized using a membrane filter prior to each use. The optimal concentration of Aureomycin in rose bengal agar, 35 ug/ml of medium, was obtained by either of two methods: (1) 0.05 ml of the stock solution was added to 10 ml of agar prior to pouring each plate, or (2) 1.0 ml of Aureomycin was added to 200 ml of medium after autoclaving. "Standard Methods" procedures (1) require pH adjustment of potato dextrose agar to 3.5 ± 0.1 with 10% tartaric acid; however, it is not necessary to adjust the pH of rose bengal agar (pH 6.0) because of the presence of Rose Bengal and Aureomycin. In some of the first studies, rose bengal agar plates were incubated at room temperature (22-30 C) for 7 days; later, for better control of incubation temperatures, plates were held in an incubator at 25 C for the same incubation time. Potato dextrose agar plates were incubated at 20 C for 5 days. At the end of incubation periods the colonies on plates of each test medium were counted and recorded, and those plates with yeasts or molds were transferred to the Fungus Studies laboratory for identification of the fungi involved. Colonies appearing on both types of media were either sight-identified or transferred to slants of neopeptone-dextrose agar for later identification studies.

The mold-type fungi were identified by the usual morphological procedures, with special attention to spore and sporophore types. Techniques for identification of yeasts are more complicated because reliance is placed not only on morphological characters of the gross colony, and the cells and cell arrangements of microcolonies growing on Dalmau slides,

but also on nutritional relationships. These relationships include growth of pure cultures on five carbon sources and three nitrogen sources in auxanogram plates, growth on 34 carbon sources including sugars, organic acids, and alcohols in replicator plates; tests of fermentation ability on glucose and, if positive, on six other sugars; temperature tolerances; and gross vitamin requirements.

Results

Since the purpose of this study was only to determine the presence or absence of a fungus colony in a dairy product, as demonstrated by one of two media, no attempt was made to determine the number of colonies arising on a plate of each species of fungus observed. During the study, 23 species of filamentous fungi and 30 species groups, including 25 identified species of yeasts, were obtained. Table ¹³⁴ / lists the various species of fungi recovered and the type of dairy product from which each species was isolated.

The numbers of fungal (or non-bacterial) colonies that appeared during the experiment were recorded as colonies per gram (or per ml) of sample (Table 135). Note that more colonies of fungi were recovered on the agar with rose bengal and antibiotics than on acidified potato dextrose agar. With some exceptions, as the colony counts increased above an estimated 1,000 per ml or g of sample (based on 33 colonies per plate of a 1:10 dilution using 10 ml of diluted sample in 3 plates), it was not possible to recognize differences in fungal colony counts from each of the two media.

Results of the physiological tests for yeast identification (i.e.,

Table 134. Species of Fungi recovered from Dairy Products.

Candida curvata (Diddens & Lodder) Lodder & Kreger - van Rij. (4)

Raw milk

Candida humicola (Daszevska) Diddens & Lodder (2)

Raw milk, cheddar cheese

Candida krusei (Castellani) Berkhout (9)

Raw milk, raw cream, raspberry flavoring for ice cream

Candida lipolytica (Harrison) Diddens & Lodder (4)

Raw milk, coffee cream (20% B.F.)

Candida mycoderma (Reess) Lodder & Kreger - van Rij. (1)

Raw milk

Candida parapsilosis (Ashford) Langeron & Talice (8)

Raw milk, raw cream, cheddar cheese, tangerine ice cream flavoring

Candida tropicalis (Castellani) Berkhout (7)

Cheddar cheese

Candida spp. (2)

Raw milk

Cryptococcus albidus (Saito) Skinner (5)

Raw milk, homogenized pasteurized milk, half and half

Cryptococcus diffluens (Zack) Lodder & Kreger - van Rij. (4)

Coffee cream (20% butter fat), homogenized pasteurized milk, skim milk

Cryptococcus laurentii (Keufferath) Skinner (2)

Raw milk

Rhodotorula glutinis (Fres.) Harrison (1)

Raw milk

Rhodotorula mucilaginosa (Jorg.) Harrison (10)

Raw milk, skim milk, coffee cream (20% b.f.)

Rhodotorula sp. (1)

Raw milk

Saccharomyces lactus Dombrowsky (Torulopsis sphaerica (Hammer & Corder)
Lodder) (10)

Cheddar cheese, raw milk

Torulopsis aerea (Saito) Lodder (3)

Raw milk, whipping cream (35% b.f.)

Torulopsis candida (Saito) Lodder (3)

Raw milk, cheddar cheese

Torulopsis dattila (Kluyver) Lodder (3)

Raw milk, raspberry ice cream flavoring

Torulopsis ernobii Lodder & Kreger-van Rij (1)

Raw milk

Torulopsis famata (Harrison) Lodder & Kreger-van Rij (3)

Raw milk

Torulopsis holmii (Jorg.) Lodder (5)

Cheddar cheese

Torulopsis inconspicua Lodder & Kreger-van Rij (11)

Raw milk, swiss cheese, cheddar cheese, coffee cream (20% b.f.)

Torulopsis stellata (Kroemer & Krombholz) Lodder (3)

Raspberry ice cream flavoring

Torulopsis spp. (6)

Mazzarella Cheese, whipping cream (35% b.f.)

Trichosporon capitatum Diddens & Lodder (1)

Raw milk

Trichosporon cutaneum (de Beurn., Gougerot & Maucher) Ota (27)

Raw milk, homogenized pasteurized milk, raw cream (b. f. unknown)

Trichosporon pullulans (Lindner) Diddens & Lodder

Raw milk

Trichosporon sericeum (Stantz) Diddens & Lodder (2)

Raw milk

Trichosporon spp. (1)

Raw milk

Alternaria tenuis Nees ss. lat. (3)

Raw milk

Ascomycete (unidentified) (1)

Homogenized, pasteurized milk

Aspergillus amstelodami (Mangin) Thom & Church (1)

Raw cream

Aspergillus chevalieri (Mangin) Thom & Church (3)

Raw milk

Aspergillus flavus Link (5)

Raw milk, ice cream stabilizer

Aspergillus fumigatus Fres. (10)

Raw milk, swiss cheese, ice cream stabilizer

Aspergillus niger van Tieghem (2)

Raw milk

Aspergillus terreus Thom (3)

Raw milk, ice cream stabilizer

Aspergillus terreus var. floccosus Shih (1)

Ice cream stabilizer

Aureobasidium pullulans (de Bary) Arnaud (2)

Raw milk

Phialophora sp. (1)

Raw milk

Cladosporium cladosporioides (Fres.) de Vries (10)

Raw milk, ice cream stabilizer

Curvularia lunata (Walkker) Boedijn (1)

Raw milk

Eremascus albus Eidam (1)

Raw milk

Fusarium oxysporum Schlecht (11)

Raw milk

Fusarium solani (Mart.) Appel & Wollen. (2)

Raw milk

Geotrichum candidum Link ex Persoon (7)

Raw milk

Paecilomyces marquandii (Mason) Hughes (1)

Raw milk

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Penicillium commune Thom (1)

Raw milk

Penicillium steckii Zaleski (1)

Raw cream

Penicillium thomii Maire (1)

Ice cream stabilizer

Phoma herbarum West (15)

(Phoma hibernica Grimes, O'Connor & Cummins)

Raw milk

Sclerotia (sterile) (1)

Raw milk

Trichoderma viride Link ex Fries (1)

Raw milk

^a Numbers added parenthetically after each species indicate number of isolates obtained. More than one isolate could have been obtained from a single sample.

^b Cultures obtained from dry skim milk solids; butter; and blue, limburger, and Camembert cheeses did not survive for final identification.

Table 135. Types of Dairy Products tested and numbers of Fungi recovered.

Number of samples	Type of samples	Number of colonies of fungi per gram of sample (Based on samples yielding highest counts)	
		on P.D.A. ^a	on R.B.A. ^b
24	Cheese		
	Natural Swiss cheese	1,500	2,200
	Provolone hard cheese	200	360
	Cheddar	450	790
	Limburger, semi-soft	5,800	11,000
	Camembert, soft	14,000,000	15,000,000
	Cheddar (midget)	10,000	11,000
	(natural)	310	350
	(brick)	100	240
	Mazzarella for Pizza	38	17.5
	Swiss, natural, aged	10,000	12,000
	Cheddar, mild	160	230
	mellow	7.5	7.5
	sharp	10	7.5
16	Butter		
	Salted butter	58,000	65,000 ^c
	Salted sweet pasteurized cream B.	1,200	1,000
	Salted creamery butter	1,300,000	1,300,000 ^d
	Salted Holland creamery butter	59,000	130,000
16	Buttermilk	Not Significant ^e	
15	Instant non-fat dry milk (1 sample)	10	340 ^f
15	Sweetened condensed milk	Not Significant ^e	
90	Miscellaneous "fresh" products		
	Whipping Cream, 1/2 pt. (35% butter fat)	170	340 ^g
19	Ice cream stabilizers and flavors		
	Tangerine ice cream flavoring	90	440 ^h
7	Ice cream stabilizers and flavors		
	Raspberry flavoring ⁱ		
	1st test (12-28-64)	120,000	130,000
	2nd test (1-4-65)	1,500,000	1,500,000 ^{1,c}
	3rd test (1-12-65)	58,000,000	58,000,000
(+35)	Raw milk, Ranges of tests of 6 samples in each of 4 series	260 - 290 210 - 870 120 - 11,000 98 - 9,500	560 - 4,600 720 - 2,200 720 - 12,000 610 - 10,000

^a Potato dextrose agar.^b Neopeptone - dextrose - rose bengal - antibiotic agar.^c Predominantly yeasts.^d Initially surface contaminated (poor packaging).^e Counts below reporting level recommended by Standard Methods.^f Other 14 samples were nonproductive. Clear halos observed around colonies.^g Other samples of homogenized milk, skim milk, half and half, chocolate dairy drink, whipping cream, and coffee cream were relatively unproductive.^h Other samples unproductive.ⁱ Sample refrigerated between tests.

carbon utilization, nitrogen utilization, and carbon fermentation tests), gave other interesting information. Of the 25 groups of strains identified to species, all used glucose; 19 used galactose; 15 used sucrose, melizitose, ethanol, D-mannitol, D-sorbitol, and Ca-2-keto-gluconate; 7 used lactose; and 4 used DL-lactic acid. Of the 7 able to ferment glucose, 5 fermented sucrose, 3 fermented galactose, maltose, and raffinose, and only one, Saccharomyces lactis, fermented lactose. Of the 25 identified species, 7 were able to grow at 37°C, 6 were able to use nitrate nitrogen, 6 were able to produce a starch-like compound in the cell wall or as a soluble substance diffusing into the surrounding medium, and 4 grew on a vitamin-free medium.

Discussion

In the examination of more than 200 samples of milk and other dairy products for filamentous fungi and yeasts, 23 species of filamentous fungi and 25 species of yeasts were recovered together with a number of unidentified strains of additional species. Since only 7 of the species utilized lactose as a carbon source and only one of these could ferment lactose, it is assumed that in general the populations recovered were casual contaminants rather than members of an indigenous population. The common milk or dairy mold, Geotrichum candidum, was found in only 7 samples of raw milk, which indicated that it may have been overlooked on many of the plates prepared with other samples of dairy products.

Interestingly, Phoma herbarum was among the commoner fungi of raw milk. This fungus is a common mold of decaying herb stems. It was first reported in the dairy-mold literature as Phoma hibernica when it was

found fairly commonly in butter in Ireland. Rhodotorula mucilaginosa, which does not utilize nitrate-nitrogen, was much more common than R. glutinis, which does. Neither, however, utilizes lactose, and no lactose-using Rhodotorula was found in this survey. The commonest species found was Trichosporon cutaneum, known to be associated with superficial mycotic infections as well as infections or contaminants of the intestinal tract. Most of these fungi do not cause severe disease in man. Several of the yeasts produce intestinal involvements of one type or another, however, especially in the tropics, and Aspergillus fumigatus strains are able to produce the pneumonia-like aspergillosis in man when spores are inhaled in quantity. A. flavus and its relatives produce the carcinogenic toxin aflatoxin. Spores of several of the filamentous fungi listed could be abundant enough in the air to produce allergenic conditions in man.

Potato dextrose agar, acidified to pH 3.3 with tartaric acid, or lactic acid has been used for many years for the primary isolation of fungi from a variety of substrates, e.g., soil, cellulosic materials, dairy products, etc. However, the effectiveness of rose bengal dye as a bacterial suppressant, as compared to acidification, has made possible the use of media at a pH of about 6.0 to 6.5 which appears to have released the germination capabilities of numerous additional fungal spores or disseminules. Since rose bengal is a vital stain, it may be expected to have some adverse effect on fungal cells, and while it has been noted that it has little or no effect on fungus spores, it has a tendency to inhibit the development of vegetative cells which lack certain protectants inherent in the spore walls.

For certain types of isolation work, a medium containing 5 g neopeptone, 10 g dextrose, 20 g agar, per liter of water, with 35 ug chlortetracycline hydrochloride (water soluble) per ml has proved to yield larger numbers of colonies of fungi from some naturally occurring materials than the medium marketed under the trade name Cooke's Rose Bengal Agar. Neopeptone dextrose agar of this formulation is also known as Emmons' or American Sabouraud Agar. This medium, when compared for growth with rose bengal agar and acidified potato dextrose agar, may prove to be more effective in the recovery or detection of fungus cells in dairy products. While the exact chemical formulation is not yet known for the various peptone products on the market, the use of one of these may yield more consistent results than the use of freshly prepared or dehydrated potato extract because of variability in potatoes obtained from various sources on each marketing expedition.

If information is desired about yeast populations, it is possible that additional species may be recovered using the techniques described in the Laboratory Guide (Cooke, 1963b). These enumeration techniques may yield quantitative data not otherwise obtainable. Since filamentous fungi are not excluded in this technique, data can also be obtained on Geotrichum candidum populations which may be present.

On the basis of colony counts obtained on primary isolations made from polluted waters and sewage treatment system samples, it has been considered by workers in the Fungus Studies Laboratory that Geotrichum candidum is one of the commoner species of fungi in the polluted water habitat. Most of these isolations have been made using neopeptone-dextrose-rose bengal-antibiotic media. Because this fungus regularly and

abundantly appears on media containing rose bengal and because it is a common contaminant of dairy products, it was surprising to find so few isolations during these studies. However, it is possible that because of our preoccupation with observation and detection of yeasts and other fungi, insufficient emphasis was given to detection of Geotrichum candidum.

All colonies picked from both media were viable through several transfers during routine laboratory storage procedures. Those colonies which were not identified soon after isolation were held for later studies. If they were held too long and dried out on agar during storage they usually could not be reactivated. In this way, several species which could have been added to the list were lost. Those cultures which were saved, because of particular interest, were forwarded to Dr. John Schmitt, Department of Botany and Plant Pathology, The Ohio State University, Columbus, when the need for a culture collection at the Fungus Studies Laboratory was no longer demonstrated.

In general, molds and yeasts in contrast to various types of bacteria, contribute little to the deterioration of dairy products. Long-range exposure to certain types of fungi, however, can cause difficulties. Such exposure could be the result of careless handling and insufficient attention to common sources of contamination such as air borne fungus spores, cells washing off or falling from animal hairs or skin, and handling of products by workers with unclean hands or equipment. Some texts do not completely identify the organisms involved, but supply brief generic characterizations of the principal isolates that may be obtained, and mention the more common species in the genera treated. Frazier (1958)

gives adequate keys for identification of the more common molds and yeasts, at least to genus and sometimes to species.

Cells and spores of yeasts and filamentous fungi usually do not survive the temperatures or the duration inherent in the usual pasteurization processes. If finished dairy products carry these organisms, therefore, such products probably were recontaminated during final processing and packaging.

E. Miscellaneous Sources of Fungal Populations

Fungi occur wherever there is a source of organic matter which can be used as food supplies. One of the greatest natural reservoirs of fungi is the soil wherein are large quantities of incompletely decomposed plant parts as well as some animal remains. More than 700 species of fungi are listed by Gilman (1957) in his "Manual of Soil Fungi." Many of these, as well as additional species, are discussed by members of a symposium whose papers were edited by Baker & Snyder (1965) on the "Ecology of soil-borne plant pathogens". That the end is not in sight is demonstrated by Raper and Fennell (1965). In this second edition of Thom and Raper's "A Manual of the Aspergilli" (1945) many of the new species are isolates from the soil.

In addition to the soil, fungi live on any surface which provides (Table 136).
food / At least a dozen species of yeasts were found by Anderson and Skinner (1947) to inhabit the surfaces and aid in the decomposition of the fleshy fruit bodies of a wide variety of mushroom-producing fungi. If not actually stated above, it has been implied that many fungi are associated with man, not only as inhabitants of his respiratory and intestinal tracts, nor as pathogens, but as organisms which occur on the

Table 156. Fungi isolated from a variety of miscellaneous environmental materials*

Species	Observation Number
<u>Trichosporon cutaneum</u>	27
<u>Cladosporium cladosporioides</u>	26
<u>Alternaria tenuis</u>	23
<u>Moniliaceae</u>	22
<u>Phoma herbarum</u>	22
<u>White yeasts</u>	20
<u>Aureobasidium pullulans</u>	18
<u>Penicillium spp.</u>	17
<u>Aspergillus fumigatus</u>	11
<u>Fusarium oxysporum</u>	11
<u>Torulopsis inconspicua</u>	11
<u>Trichoderma viride</u>	11
<u>Mucor spp.</u>	10
<u>Rhodotorula muciliaginosa</u>	10
<u>Saccharomyces lactis</u>	10
<u>Candida krusei</u>	9
<u>Penicillium lilacinum</u>	9
<u>Candida parapsilosis</u>	8
<u>Geotrichum candidum</u>	8
<u>Rhodotorula spp.</u>	8
<u>Aspergillus flavus</u>	7
<u>Candida tropicalis</u>	7
<u>Torulopsis spp.</u>	6
<u>Cephalosporium spp.</u>	5
<u>Phoma spp.</u>	5
<u>Torulopsis holmii</u>	5
<u>Aspergillus sp.</u>	4
<u>Aspergillus niger</u>	4
<u>Candida curvata</u>	4
<u>Candida lipolytica</u>	4
<u>Cryptococcus albidus</u>	4
<u>Cryptococcus diffluens</u>	4
<u>Fusarium solani</u>	4
<u>Phialophora jeanselmei</u>	4
<u>Rhizopus sp.</u>	4
<u>Sphaeronema sp.</u>	4
<u>Aspergillus terreus</u> var. <u>terreus</u>	3
<u>Curvularia lunata</u>	3
<u>Fusarium roseum</u>	3
<u>Gliocladium catenulatum</u>	3
<u>Gliocladium roseum</u>	3
<u>Penicillium commune</u>	3
<u>Pestalotia heterocornis</u>	3
<u>Stemphylium sp.</u>	3
<u>Torulopsis aeria</u>	3
<u>Torulopsis candida</u>	3
<u>Torulopsis datilla</u>	3
<u>Torulopsis famata</u>	3
<u>Trichosporon sp.</u>	3
<u>Aspergillus chevalieri</u>	2
<u>Aspergillus versicolor</u>	2
<u>Candida sp.</u>	2
<u>Candida humicola</u>	2
<u>Choanephora trispore</u>	2
<u>Cryptococcus laurentii</u>	2
<u>Isaria farinacea</u>	2

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<u>Phialophora</u> spp.	2
<u>Rhodotorula</u> <u>glutinis</u>	2
<u>Torulopsis</u> <u>stellata</u>	2
<u>Trichosporon</u> <u>sericeum</u>	2

The following species were only observed once in this series of isolations:

Aspergillus amstelodami, A. terreus var. floccosus, Candida mycoderma,
Dichotomyces albus, Epicoccum purpurascens, Eremascus albus, Fusarium sp.,
F. aqueductum, Monotospora daleae, Mucor fragilis, Myrothecium verrucaria,
Paecilomyces fumoso-roseus, P. marquandii, Pennicillium funiculosum, P. herquesi,
P. ochrochloron, P. oxalicum, P. thomii, P. urticae, P. viridicatum, Sclerotium sp.,
Stemphylium botryosum, Torulopsis ernobii, Trichosporon capitatum, T. pullulans,
Verticillium sp., V. lateritium.

*Samples from which these species were obtained include:

Samples of milk and milk products described in Chapter 14
Litter from Hyde Park Country Club used in study of phenols in soil runoff water
Sample of melted ice blocks which fell in a Philadelphia suburb in late spring
Samples of slimes from wells
A sample of red snow from 10,750 ft. in the Rocky Mountains, Colorado
Samples of water and algae in thermal springs, Wyoming
Scrapings from inside a house drain, Cincinnati
Scrapings of paint from a paint-mold infested house, Cincinnati
Samples of cutting oils, Cincinnati
Samples of skin from a foot, Cincinnati

surface of his body. In this connection, the yeasts have received some attention(Connell & Skinner, 1953; Connell, Skinner & Hurd, 1954; Huxley & Hurd, 1956; Skinner & Huxley, 1956). In a less personal way, the fungi are also associated with the clothing of travelers. In a series of swabs taken from the shoes of passengers arriving at the Honolulu International Airport, and at docks and other airfields, it has been shown that at least 65 species of fungi have become opportunistic travelers ("versatile opportunists") (Baker, 1966).

Not only in high-altitude sampling of the air, but also in the comparative quiet of the laboratory, it is abundantly demonstrated that viable fungal spores are present at all times. A wide variety of laboratory solutions from potent acids to mineral solutions, from carefully prepared standard solutions to inks used at the desk, molds are occasional nuisances. In such cases the same species which may be a useful agent of organic matter decomposition in sewage, is a distinct nuisance in the no-longer standard solution in the laboratory, or a useful marker of air currents in the upper air.

XV. The Role of Fungi in Environmental Sanitation*

Fungi are common organisms that occur abundantly in nature wherever decaying organic matter is found. These organisms cannot manufacture their own food supplies and, therefore, must depend on other preformed organic matter as a source of nutrient supplies. Thus, fungi are scavengers or decomposers.

In the world of living things from the point of view of ecological relationships (Whittaker 1957, 1959), there are three basic types of

*Reproduced with permission from Developments in Industrial Microbiology, Plenum Press, New York 3:313-318, 1962.

organisms, the producers, the consumers, and the decomposers. The producers, through the use of sunlight, manufacture materials that supply the basic needs of the other groups. These organisms have chlorophyll or chemosynthetic pigments that, in the presence of light, change water and carbon dioxide into sugars and other substances. These substances become the food of man when elaborated, his shelter when he builds his home of wood, his means of communication when he sends a letter or reads a newspaper. The consumers use the products of the producer organisms as food, ingesting these products and discarding the indigestible materials. All animals, from the protozoan which accepts the smallest bacterial cell as food, to man, who ingests green salad, cooked vegetables, or meat products from herbivorous animals, are consumers. The decomposers, which include fungi, accept the products from the activities of both the producers and the consumers, reducing them to the original elements from which they are fashioned into organic matter by the producers.

The most abundant groups of decomposer organisms include the bacteria and the fungi. Except for obligate plant pathogens, all known fungi are decomposers in nature. Simplified, the ecological life of a soil fungus (Garrett, 1956) starts with a spore lying on or in the ground. A piece of organic matter washes to this spore, having floated down to it as a piece of leaf onto the ground, or grown past it as a root tip or stem sprout. The new source of nutrients activates the germination of the spore, which then develops in such a way that it takes advantage of the new food supply. When this food supply is exhausted the fungus returns

to a resting state by producing a new crop of spores. The spores may be blown great distances. Viable spores that originated in the tropics have been recovered from air masses over Canadian regions (Kelly & Pady, 1953), and viable spores have been recovered from the deepest troughs of the Pacific Ocean.

If a spore is washed to a stream from the soil of a field or from a woodland by the runoff from a rainstorm, and if this stream carries a load of nutrients the spore can assimilate, then the spore will germinate. A thallus will be formed around a grain of organic or inert matter, and so long as it is bathed in this nutrient material it will grow and may even produce more spores. During its sojourn in the more heavily enriched portions of the stream, this organism thus contributes to the reduction of pollution in the stream.

The airborne spore may settle out of the air onto the soil, the roof top, the sidewalk, or some other handy surface, or may be washed out of the air in a rainstorm. Spores are present in numbers in the dirt on hands that have made mud pies or have tended the garden; they also may be present in large numbers in the soil around root crops or on the surfaces of things being washed, such as floors, windows, clothing, etc. All these spores are brought together in the household drainage system, from which they are passed on to the sewer, and eventually to the sewage treatment system. Here some transients may be weeded out because they cannot adapt to the liquid environment, whereas others remain in this new habitat and are bathed continually in an environment in which adequate food materials are present or are released through the activity of other organisms.

In the economy of nature, fungi are useful members of any population. They degrade the remains of once living plants and animals, reducing these substances to the elements from which they were produced. Of course, bacteria and other scavenger organisms aid in the process. In the economy of man, the degrading activities of molds usually have been considered detrimental, as in the loss of millions of dollars worth of cellulosic and plastic supplies in tropical areas during World War II. In industries that manufacture merchantable materials, the specialist in the prevention of deterioration is important. He tries to find ways of preventing the deterioration of telephone poles, railroad ties, cotton cloth, plastic equipment, optical equipment, paints, and other crucial supplies.

In the economy of man we are in the habit of thinking of yeasts in one of two ways: yeast that produces alcohol and carbon dioxide as a result of fermenting natural sugars, thus producing beer, whiskey, wine, and other alcoholic products, as well as making bread rise; and yeast that can be turned into great masses of food for man or for his cattle by the utilization of various types of naturally occurring or modified sugars, such as those in spent sulphite liquor. Except in the food industry, we do not think of yeasts as causing difficulties in dried fruits such as prunes, raisins, and apricots, or in stored fruits such as olives, pickles, etc.

From this brief review we can derive a principle of nature: Wherever there is natural organic matter, there also is a fungal organism capable of aiding in the reduction of the matter to its simplest components.

Can we put this principle to work for us in the removal of organic wastes just as the specialist in the prevention of deterioration tries to stop its action?

It has been shown that in the polluted streams and in the secondary-type sewage treatment plants, both trickling filter and activated sludge types, large numbers of decomposer-type organisms are basic members of the population. Until recently it was thought that these organisms were largely bacteria and protozoans. Studies on the ecology of these population systems have shown that definite food chains exist and that they operate in much the same way in polluted streams and secondary-type sewage treatment plants as in other habitats.

Basically, the decomposers include bacteria, filamentous fungi, and yeasts. They may assimilate any dissolved materials that pass over them or in which they are floating or swimming, or they may adsorb or possibly trap colloidal or solid fragments for delayed assimilation. Within the total populations are large numbers of consumers, amoeboid and flagellated protozoans, that are bathed continually in this supply of dissolved and colloidal food materials. These consumers, depending on their size, are capable of ingesting any organism from small bacteria to large fungal cells. Also in the population system, within the niche they can occupy, are pigmented organisms, algae. These producers may consume some of dissolved organic matter in the sewage, but primarily they add oxygen to the system during the daylight hours. Algae are the only producers found in the population system. Small protozoans may be consumers of bacteria; larger protozoans are consumers of bacteria and individual fungus cells, as are metazoans. Feeding on the whole population are large

quantities of fly larvae, annelid worms, and snails.

Up to the present time, such composite populations of organisms in sewage treatment systems, like Topsy, have just grown. In a trickling filter, a pile of rocks is placed in a tank and a liquid is poured over these rocks. An organism that is adaptable to such a habitat comes along, pauses, decides it likes the location, and settles down to become a member of the population, which first is scattered but later is homogenous. To date, no attempt has been made to control this development beyond the superstitious addition of a yeast cake here, a shot of enzyme there, or a shovel-ful of horse manure over there.

As a result of studies of deterioration of a large number of substances, especially of such natural plant products as cellulose in the form of cotton fabric (White et al., 1948; Siu, 1951), rope, tentage, wood products, linen and other materials, and of such natural plant products as keratin-containing wool cloth (White et al., 1950), we know of some organisms that are capable of degradation. A practically limitless number of bacteria, filamentous fungi, and yeasts are known to be involved in one type or another of degradation of food substances, from raw produce in the field, in storage, in transit, in the market, or in the home or restaurant, to preserved food, especially home-canned vegetables, fruits, jams, or jellies. From daily experience we know, or from occasional observation we are aware, that some bacteria, yeasts, and molds are capable of attacking meats and other food products in storage between the producer plant or animal and the consumer human.

In our laboratory it has been found that 8 of the 30 or more common

filamentous fungi of sewage, sewage-polluted water, and sewage treatment systems are capable of degrading simple to complex carbon and nitrogen sources, since these substances are used as nutrients and growth takes place in their presence. We have found that they are self sufficient for vitamins and that they can utilize oxygen when it is present only in the dissolved state and other organisms are competing for it. We have found that a few of these organisms are capable of growing when such hydrocarbons as kerosene or motor oil (SAE 20) are the only available sources of carbon.

We have found that on a high-rate and a low-rate trickling filter, filamentous fungi form the matrix of slime in which other organisms are imbedded, through which other organisms are able to move about in search of food, and over which other organisms move in the process of grazing. We have found that the same group of common fungi is present in every type of sewage-polluted habitat that has been tested: in sewage, in sewage-polluted streams, in trickling-filter and activated-sludge sewage treatment plants, and in waste stabilization ponds. Can such populations or the organisms in such population systems be put to work for us?

In man's developing civilization, many products have been invented and produced that are formed of substances different from those found in nature. When the waste products from this type of manufacture are released into a stream, they become potential nutrients for whatever organisms are present. The simpler compounds, those nearer in formulation to naturally occurring compounds, are attacked by one or another micro-organism and picked apart. One organism may be able to use one fraction,

a second another fraction, and a third may utilize the waste products produced by either or both of the first two. When a byproduct of a totally different formulation than those even rarely found in nature finds its way to such a population of microorganisms, however, nothing in particular happens to it; before it is available to any microorganism, it must go through some other form of degradation.

This seems to be the fate of alkyl-benzyl-sulfonates (ABS), which form a large part of the basic chemicals in hard detergents. ABS does not seem to be available to those kinds of fungi to which it has been exposed in the laboratory (but see Chapter XII-E above), and at low concentrations it is not toxic to the fungi. Simply, ABS appears not to be in the environment of the fungus at all; however, other compounds in the same solution are available to the fungus.

In the forest, a number of fungi occur that are able to use lignin, which is present in the woody tissues of all vascular plants. Even herbs contain a small amount of this strength-giving cementing substance. Whether it is in chemical or physical union with the cellulose of the stem or leaf is unknown or undecided. Some mushroom-bearing fungi can attack one of these two substances in leaf litter, others can attack that in the stems, and a few can attack both (Lindeberg, 1944). In a standing tree, a large number of species of bracket-fungi can utilize the lignin of the stem, but a larger number attack the cellulose. Trees fall to the forest floor with other kinds of litter, and if lignin-decomposing organisms - mostly fungi - and cellulose-attacking organisms were not present, the earth's surface would be covered by a deep layer of undecomposed

plant remains. These organisms work rapidly, however, as time is measured by nature. Much of the cellulose is decayed by the end of the first year after leaf fall, and much of the lignin is removed by the third year, leaving only humic acids (Burgess, 1958; Burgess & Latter, 1960). No one knows much about humic acids yet (Johnston, 1961), but it is known that they are highly resistant to decay processes. A soil rich in humic acids is a soil rich in nutrients, as anyone who uses composts, leaf mulch, and black woodland soil in his flower beds knows. However, it takes a long time to decompose these materials in place, and their fate when moved to another place or when a new field is plowed is unknown.

It would seem reasonable to expect to be able to put fungi, whose normal nutrients are found in lignins in wood and leaf litter, to work cleaning up the lignin-bearing waste waters discharged from paper and rayon mills. But so far this is an idle expectation. At least four different groups of workers (Adams and Ledingham, 1942; Day et al., 1953; Raynaud et al., 1956, and this laboratory) to date have subjected lignin or lignosulfonic-acid-containing materials to fungal or bacterial action, and all these workers have arrived at the same ultimate conclusion. During the earlier stages of experimentation the lignin-containing compounds appear to be removed from solution. A certain amount of growth takes place. The growth appears to be brown in color. Optical examination of the surface of the fungal mycelium or the bacterium shows, however, that the lignin is only adsorbed onto the surface of the mycelium and is not assimilated by the organism. Chemical and physical examinations show that about the same amount of lignin-containing substance is present outside

organism as was in solution at the beginning of the experiment.

One paper mill has found a way to dispose of some of its wastes by spraying spent sulphite liquor on a nearby field (see Chapter IX-B). This field is essentially a 90 ft. deep bed of glacial sand and gravel in a preglacial canyon. It forms an excellent trickling filter sand bed. This sand bed had been useless even for planting a tree farm, because water was not held long enough to allow seedlings or saplings of pulp-producing trees to get started. After three years of use for this type of waste disposal, however, the water holding capacity of the top 20 ft. of the soil had improved considerably, a larger number of microorganisms had been able to develop, and the sand bed had filtered out most of the BOD poured on it. Also, lignin-like compounds had remained in the soil, probably forming an increasing amount of humus-like material.

Recent studies in England (Henderson, 1960), as yet unconfirmed elsewhere and ~~h~~ completely reported by the originating laboratory, indicate that it is possible that, in the laboratory, natural lignins may be decomposed at first by wood and litter decay organisms that initiate the breakdown of the lignin complex into the several aromatic compounds from which it was built up. These compounds in turn would be decomposed by filamentous soil fungi into compounds without ring structure, and would be readily available to the same group or to other organisms in the habitat. This is a slow process, since the microorganisms involved are not familiar with production-line speedups, piece-work bonuses, and other methods of getting a job done quickly.

The training of microorganisms to attack one or another type of natural or synthetic chemical is a task that has not been given much attention.

People in the production business know techniques for inducing greater production of purer chemicals by microorganisms, both bacterial and fungal. These techniques involve the manipulation of the genetic factors inherent in the culture under consideration. Fungi of true sex but capable of asexual heterocaryotic reproduction, and even of parasexuality, are known. In all four of these basic reproductive types, the nuclear condition can be altered by mutagenic agents in such a way that the physiology of the organism may be permanently changed, or the organism may be adapted to a particular situation, generation after generation. In either event the changes brought about may lead to a technique for the reduction of any chemical by-product that causes pollutional problems. This is the reverse of the activity of a specialist in the prevention of deterioration, who tries to find ways to prevent the deterioration of a commercially valuable product.

APPENDIX

A list of more than 500 species of fungi is appended to this report (Table 137). These species have been isolated in various projects to determine the extent of occurrence and the populations of fungi involved in various aspects of environmental sanitation, sewage pollution, deterioration of materials in relation to organisms which may be pathogenic, etc. A systematic guide to these fungi, including data on saprobic valency and other factors of interest, is being prepared.

Table 137 Summary list of fungi isolated from environmental samples described above. Listed alphabetically by species in major morphological groups with total observation numbers.

Species	Observation number
Aquatic Phycomycetes	
<u>Achlya</u> spp.	22
<u>americana</u>	18
<u>debaryana</u>	3
<u>diffusa</u>	4
<u>dubia</u>	11
<u>flagellata</u>	3
<u>glomerata</u>	2
<u>klebsiana</u>	16
<u>prolifera</u>	1
<u>Alloxyces</u> spp.	7
<u>arbuscula</u>	4
X <u>javanicus</u>	2
<u>macrogya</u>	1
<u>Aphanomyces</u> spp.	26
<u>laevis</u>	4
<u>scaber</u>	1
<u>Dictyuchus</u> spp.	10
<u>monosporus</u>	7
<u>Leptomitum lacteus</u>	1
<u>Pythium</u> spp.	98
<u>Rhizophidium</u> sp.	6
<u>Saprolegnia</u> spp.	27
<u>delica</u>	8
<u>ferax</u>	10
<u>littoralis</u>	1
<u>Thraustotheca clavata</u>	1
<u>Phycomycetes</u> spp.	14
Colorless algae	
<u>Prototheca moriformis</u>	1
<u>stagnora</u>	13
Mucorales	
<u>Absidia californica</u>	2
<u>corymbifera</u>	7
<u>cylindrospora</u>	4
<u>glauc</u>	5
<u>ramosa</u> var. <u>ramosa</u>	16
<u>biappendiculata</u>	3
<u>spinosa</u>	10
<u>Actinomucor elegans</u>	8
<u>Choanephora cucurbitarum</u>	1
<u>trispore</u>	2
<u>Circinella umbellata</u>	2
<u>Conidiobolus villosus</u>	4
<u>Cunninghamella bainieri</u>	1
<u>echinulata</u>	2
<u>elegans</u>	10
<u>Gilbertella persicaria</u>	1
<u>Gongronella butleri</u>	8
<u>Mortierella</u> sp.	2
<u>isabellina</u>	3
<u>Mucor</u> spp.	814
<u>alternans</u>	90
<u>angulisporea</u>	44

Table p.2

<u>Mucor</u> <u>brunneus</u>	1
<u>corticola</u>	4
<u>dispersus</u>	1
<u>?foenicola</u>	2
<u>fragilis</u> Sect.	1
<u>fragilis</u>	575
<u>globosus</u>	6
<u>hiemalis</u> Sect. n.sp. Hes.	25
<u>hiemalis</u>	1415
nr. <u>Jensenii</u>	1
<u>mucedo</u>	1
<u>petropolitanus</u> Sect.	1
<u>plumbeus</u>	156
<u>pranii</u>	1
<u>racemosus</u>	40
nr. <u>saturninus</u>	10
<u>subtilissimus</u>	6
<u>Piptocephalis</u> <u>lemonnierianus</u>	1
<u>Rhizopus</u> spp.	294
<u>sp. parasitized</u>	5
<u>arrhizus</u>	75
<u>chinensis</u> Ser.	7
<u>microsporus</u>	1
<u>nigricans</u>	6
<u>oryzac</u>	4
<u>rhizopodiformis</u>	22
<u>stolonifer</u>	1
<u>Syncephalastrum</u> <u>racemosum</u>	31
<u>Zygorhynchus</u> <u>moelleri</u>	95
<u>heterogamicus</u>	1
Ascomycetes	
<u>Arachniotus</u> <u>citrinus</u>	1
<u>Ascodesmis</u> <u>sphaerospora</u>	1
<u>Chaetomium</u> spp.	5
<u>brasiliense</u>	1
<u>cancroideum</u>	4
<u>funiculosum</u>	6
<u>globosum</u>	63
<u>Dichotomyces</u> <u>albus</u>	1
<u>Emericellopsis</u> <u>humicola</u>	11
<u>synnemata</u>	5
<u>Eremascus</u> <u>albus</u>	1
<u>Nectria</u> <u>flavo-virens</u> (stat. conid.)	1
<u>Preussia</u> <u>dispersa</u>	3
<u>microspora</u>	1
<u>Pseudogymnoascus</u> <u>roseus</u>	1
<u>Pseudoplea</u> <u>americana</u>	2
<u>?Sordaria</u> sp.	1
<u>Subbaromyces</u> <u>splendens</u>	35
<u>Talaromyces</u> <u>spiculosporus</u>	3
<u>ucrainicus</u>	2
<u>?Xylaria</u> sp.	1
<u>Gymnoascus</u> <u>violaceus</u>	2
<u>Thielavia</u> sp.	4
<u>sepedonium</u>	2
Ascomycetes	6
Pyrenomycetes	1

Table 137 P. 3

Basidiomycetes	
<u>Polyporus versicolor</u>	
<u>Poria ambigua</u>	
Basidiomycetes	1
Yeasts (Perfect and Imperfect)	2
<u>Candida</u> spp.	2
<u>albicans</u>	
<u>catenulata</u>	69
<u>curvata</u>	6
<u>guilliermondii</u>	43
<u>humicola</u>	115
<u>intermedia</u>	128
<u>japonica</u>	67
<u>krusei</u> (incl. some <u>lambica</u>)	72
<u>lambica</u>	4
<u>lipolytica</u>	819
<u>lusitana</u>	13
<u>melinii</u>	4
<u>mesenterica</u>	3
<u>mycoderma</u>	1
<u>parapsilosis</u>	4
<u>pelliculosa</u>	30
<u>pseudotropicalis</u>	386
<u>pulcherrima</u>	3
<u>ravanti</u>	3
<u>robusta</u>	25
<u>rugosa</u>	1
<u>scottii</u>	2
<u>solani</u>	18
<u>tropicalis</u>	3
<u>utilis</u>	15
<u>zeylanoides</u>	311
<u>Cryptococcus</u> spp.	30
<u>albidus</u>	2
<u>diffusus</u>	16
<u>laurentii</u>	51
<u>luteolus</u>	37
<u>Debaryomyces nicotinae</u>	82
<u>Endomyces</u> n.sp. Cain	16
<u>Endomyces</u> <u>javanensis</u>	1
<u>Hansenula</u> spp.	1
<u>anomala</u>	11
<u>californica</u>	5
<u>canadensis</u>	14
<u>ellipsoidospora</u>	16
<u>saturnus</u>	2
<u>suaevolens</u>	3
<u>Kloeckera apiculata</u>	17
<u>Pichia fermentans</u>	1
<u>guilliermondii</u>	33
<u>membranefaciens</u>	5
<u>Rhodotorula</u> spp.	12
<u>flava</u>	8
<u>glutinis</u> var. <u>glutinis</u>	860
<u>rubescens</u>	2
<u>gravinis</u>	281
<u>macerans</u>	4
<u>minuta</u>	3
<u>mucilaginea</u>	1
	3
	491

Table 137 p.4

<u>Rhodotorula</u> <u>pennaeus</u>	3
<u>pilimanae</u>	32
<u>rubra</u>	1
<u>texensis</u>	108
<u>Saccharomyces</u> spp.	21
<u>carlsbergensis</u>	1
<u>cerevisiae</u> var. <u>cerevisiae</u>	151
<u>tetrasporus</u>	1
<u>drosophilae</u>	2
<u>fructum</u>	2
<u>heterogamicus</u>	1
<u>lactis</u>	10
<u>montanus</u>	2
<u>rossi</u>	1
<u>Schwanniomyces</u> <u>alluvius</u>	3
Smut-like	32
<u>Sporobolomyces</u> <u>roseus</u>	1
<u>Torulopsis</u> spp.	105
<u>aeria</u>	24
<u>candida</u>	263
<u>carpophila</u>	2
<u>colliculosa</u>	4
<u>dattila</u>	23
<u>ernobii</u>	2
<u>femata</u>	310
<u>glabrata</u>	153
<u>holmii</u>	14
<u>inconspicua</u>	90
<u>?saki</u>	16
<u>stellata</u>	22
<u>vanzylii</u>	9
<u>versatilis</u>	7
<u>Trichosporon</u> spp.	153
<u>behrendii</u>	3
<u>capitatum</u>	53
<u>cutaneum</u> var. <u>cutaneum</u>	311
<u>multisporum</u>	2
<u>fermentans</u>	3
<u>margaritifera</u>	3
<u>pullulans</u>	29
<u>sericeum</u>	4
Unidentified yeasts	1719
<u>Phomaceae</u>	
<u>Ascochyta</u> sp.	1
<u>Chaetomella</u> sp.	3
<u>Coniothyrium</u> spp.	64
<u>fuckelii</u>	46
<u>Cytospora</u> sp.	3
<u>Diplodia</u> <u>seae</u>	1
<u>Diplodina</u> sp.	1
<u>Phoma</u> spp.	889
<u>herbarum</u>	82
<u>terrestris</u>	2
<u>Pyrenochaeta</u> sp.	4
<u>Selenophoma</u> sp.	1
<u>Septoria</u> sp.	1
<u>Sphaeronema</u> sp.	5
<u>spinella</u>	15
<u>Sphaeropsis</u> sp.	2

Table 137 p. 5

Melanconiales

<u>Colletotrichum gloeosporioides</u>	10
<u>Gloeosporium</u> sp.	2
<u>Pestalotia</u> sp.	15
<u>dichaeta</u>	3
<u>heterocornis</u>	13
<u>versicolor</u>	3
<u>Monochaetia kansanensis</u>	1

Moniliales

<u>Acremonium</u> <u>alternatum</u>	9
<u>Acrospira</u> <u>levis</u>	5
<u>Alternaria</u> <u>tenuis</u>	624
<u>Arthrinium</u> <u>arundinis</u>	1
<u>Arthrobotrya</u> sp.	7
<u>muscaeformis</u>	3
<u>oligospora</u>	1
<u>Aspergillus</u> spp.	164
<u>amstelodami</u>	83
<u>candidus</u>	95
<u>carneus</u>	20
<u>chevalieri</u> var. <u>chevalieri</u>	94
<u>intermedius</u>	7
<u>clavatus</u>	47
<u>fischeri</u>	5
<u>flavipes</u>	76
<u>flavus</u>	387
<u>fumigatus</u>	546
<u>luchuensis</u>	2
<u>melles</u>	1
<u>nidulans</u> var. <u>nidulana</u>	6
<u>latus</u>	1
<u>niger</u>	646
<u>ochraceus</u>	264
<u>oryzae</u>	1
<u>repens</u>	1
<u>restrictus</u>	32
<u>ruber</u>	8
<u>sydowii</u>	198
<u>tamarii</u>	30
<u>terreus</u> var. <u>terreus</u>	74
<u>floccosus</u>	9
<u>unguis</u>	7
<u>ustus</u> var. <u>ustus</u>	93
<u>laevia</u>	5
<u>versicolor</u>	518
<u>ventii</u>	21
<u>Aureobasidium</u> <u>mansonii</u>	3
<u>pullulans</u>	1134
<u>Beauveria</u> <u>bassiana</u>	10
<u>Botryotrichum</u> <u>piluliferum</u>	10
<u>Botrytis</u> sp.	1
<u>cinerea</u>	4
<u>terrestris</u>	1
<u>Calcarisporium</u> <u>arbusculum</u>	1
<u>Cephalosporium</u> spp.	545
<u>coremioides</u>	15
<u>Chloridium</u> <u>chlamydosporum</u>	11
<u>Chrysosporium</u> <u>pannorum</u>	8

Table 137 p. 6

<u>Cladosporium</u> spp.	28
<u>avellaneum</u>	7
<u>cladosporioides</u>	1167
<u>elatum</u>	1
<u>herbarum</u>	52
<u>macrocarpum</u>	2
<u>sphaerospermum</u>	7
<u>Coccospora</u> sp.	2
<u>Curvularia</u> spp.	46
<u>geniculata</u>	11
<u>intermedia</u>	1
<u>lunata</u>	21
<u>Cylindrocarpum radicicola</u>	2
<u>Cylindrosporium</u> sp.	1
<u>Dendryphium</u> sp.	2
<u>Diplocladium</u> sp.	1
<u>Doratomyces microsporus</u>	1
<u>stemonitis</u>	32
<u>Echinobotryum atrum</u>	1
<u>Epicoccum purpurascens</u>	257
<u>Fusarium</u> spp.	151
<u>acuminatum</u>	64
<u>aquaeductum</u>	789
<u>avenaceum</u>	2
<u>chlamydosporum</u>	2
<u>conglutinans</u>	8
<u>culmorum</u>	3
<u>dimerum</u>	1
<u>equiseti</u>	2
<u>graminearum</u>	18
<u>moniliforme</u> var. <u>moniliforme</u>	15
<u>subglutinans</u>	2
<u>oxysporum</u> var. <u>oxysporum</u>	599
<u>redolens</u>	1
<u>poae</u>	2
<u>roseum</u> (including <u>sensu lato</u>)	76
<u>sambucinum</u> var. <u>sambucinum</u>	4
<u>caeruleum</u>	1
<u>semitectum</u>	2
<u>solani</u>	140
<u>tricinctum</u>	1
<u>Fusidium viride</u>	3
<u>Geotrichum candidum</u>	2409
<u>Gilmanella bumicola</u>	6
<u>Gliocladium</u> spp.	38
<u>catenulatum</u>	31
<u>deliquescent</u>	27
<u>fimbriatum</u>	25
<u>penicillatum</u>	3
<u>roseum</u>	312
<u>Gliomastix</u> sp. (white)	1
<u>convoluta</u>	47
<u>Gonatobotrys</u> sp.	1
<u>Gonytrichum macrocladium</u>	7
<u>Graphium</u> sp.	4
<u>Haplographium</u> sp.	3
<u>Helicomyces liliputicus</u>	1
<u>Helminthosporium</u> sp.	8
<u>Humicola grisea</u>	10
<u>Hymenula</u> sp.	1
<u>Isaria farinacea</u>	6

Table 157 p. 7

<u>Keratinomyces</u> <u>ajelloi</u>	1
<u>Leptographium</u> sp.	27
<u>Libertella</u> sp.	1
<u>Mesmonia</u> <u>echinata</u>	41
<u>Menispora</u> sp.	1
<u>Monilia</u> sp.	2
<u>sitophila</u>	20
<u>Monosporium</u> <u>apiospermum</u>	48
<u>olivaceum</u> var. <u>major</u>	2
<u>sylvaticum</u>	1
<u>Monotospora</u> sp.	2
<u>daleae</u>	1
<u>humicola</u>	1
<u>Myrothecium</u> <u>verrucaria</u>	105
<u>Nigrospora</u> <u>sphaerica</u>	6
<u>Oidiobondron</u> spp.	14
<u>Oospora</u> <u>lupuli</u>	1
<u>Pachybasium</u> sp.	2
<u>Paecilomyces</u> spp.	112
<u>elegans</u>	11
<u>fumoso-reseus</u>	2
<u>marquandii</u>	16
<u>persicinus</u>	1
<u>variotti</u>	173
<u>Papulospora</u> sp.	1
<u>Penicillium</u> spp.	3710
<u>aculeatum</u>	1
<u>adametzii</u>	1
nr. <u>aurantiacum</u>	2
<u>aurantio-violaceum</u>	8
<u>aurantio-virens</u>	2
<u>brefeldianum</u>	4
<u>brevicompactum</u>	7
<u>canescens</u>	12
<u>capsulatum</u>	1
<u>carminc violaceum</u>	1
<u>casei</u>	5
<u>charlesii</u>	29
<u>chrysogenum</u>	200
<u>citreo-viride</u>	2
<u>citrinum</u> f. 2	5
<u>claviforme</u>	2
<u>clavigerum</u>	7
<u>commune</u>	39
<u>corylophilum</u>	7
<u>corymbiferum</u>	24
<u>crustosum</u>	9
<u>cyaneofulvum</u>	4
<u>cyclopium</u> var. <u>cyclopium</u>	19
<u>echinulatum</u>	1
<u>daleae</u>	2
<u>decumbens</u>	9
<u>digitatum</u>	1
<u>diversum</u>	4
<u>duclauxii</u>	1
nr. <u>ehrlichii</u>	1
<u>expansum</u>	73
<u>fellutanum</u>	13
<u>frequentens</u>	18
<u>funiculosum</u>	656
<u>gladioli</u>	3

Table 137 p. 8

<u>Penicillium</u>	<u>granulatum</u>	1
	<u>herquei</u>	84
	<u>implicatum</u>	16
	<u>islandicum</u>	3
	<u>italicum</u>	1
	<u>janthinellum</u>	376
	<u>javanicum</u>	50
	<u>jensenii</u>	3
	<u>kapuscinskii</u>	18
	<u>lanatum</u>	1
	<u>lanosum</u>	25
	<u>levitum</u>	4
	<u>lilacinum</u>	1435
	<u>lividum</u>	2
	<u>luteum</u>	9
	<u>martensii</u>	106
	<u>melagrinum</u>	1
	<u>multicolor</u>	3
nr.	<u>nalgeovensis</u>	1
	<u>nigricans</u>	79
	<u>notatum</u>	1
	<u>ochrochloron</u>	582
	<u>oxalicum</u>	190
	<u>palitans</u>	14
	<u>pallidum</u>	1
	<u>parvum</u>	5
	<u>piceum</u>	1
	<u>piscarium</u>	2
	<u>psittacinum</u>	1
	<u>puberulum</u>	28
	<u>pulvillorum</u>	17
	<u>purpureogenum</u> var. <u>purpureogenum</u>	98
	<u>rubri</u>	1
	<u>pusillum</u>	1
	<u>raistrickii</u>	11
	<u>restrictum</u>	4
	<u>rolfsii</u>	2
	<u>roquefortii</u>	6
	<u>roseo-purpureum</u>	1
	<u>rubrum</u>	28
	<u>rugulosum</u>	7
	<u>sclerotiorum</u>	15
	<u>simplicissimum</u>	20
	<u>solitum</u>	9
	<u>spinulosum</u>	8
	<u>steckii</u>	4
	<u>stipitatum</u>	15
	<u>stoloniferum</u>	6
	<u>tardum</u>	1
nr.	<u>terlikowskii</u>	1
	<u>terrestris</u>	2
	<u>thomii</u>	60
	<u>turbatum</u>	1
	<u>urticae</u>	2
	<u>variabile</u>	214
	<u>varians</u>	1
	<u>velutinum</u>	45
	<u>vermiculatum</u>	14
	<u>viridicatum</u>	5
	<u>yakmanii</u>	5

Table 137 p. 9

<u>Periconia</u> sp.	8
<u>Peyronellea</u> sp.	5
<u>Phaeoisaria</u> sp.	2
<u>Phialocephala</u> sp.	4
<u>dimorphospora</u>	7
<u>heterospora</u>	1
<u>Phialophora</u> spp.	37
<u>fastigiata</u>	5
<u>jeanselmei</u>	1215
<u>lagerbergii</u>	2
<u>richardsiae</u>	1
<u>Rhinocladiella</u> <u>atrovirens</u>	1
<u>Sclerotium</u> spp.	1
<u>rolfsii</u>	2
<u>Scopulariopsis</u> spp.	4
<u>asperula</u>	3
<u>brevicaulis</u>	14
<u>brumptii</u>	1
<u>canadense</u>	2
<u>chartarum</u>	2
<u>parvula</u>	2
<u>Sepedonium</u> sp.	21
<u>thielavioides</u>	1
<u>Sporidesmium</u> <u>bakeri</u>	1
<u>Sporotrichum</u> spp.	16
<u>epigaeum</u> var. <u>terrestre</u>	3
<u>laxum</u>	1
<u>pruinsum</u>	11
<u>?schenckii</u>	3
<u>Stachybotrys</u> <u>atra</u>	74
<u>Stemphylium</u> spp.	33
<u>botryosum</u>	13
<u>consortiale</u>	22
<u>lanuginosum</u>	3
<u>Stilbella</u> <u>bulbicola</u>	2
<u>Stysanus</u> sp.	1
<u>Tetracoccosporium</u> <u>paxianum</u>	3
<u>Thysanophora</u> sp.	1
<u>penicilloides</u>	1
<u>Torula</u> sp.	3
<u>herbarum</u>	1
<u>Trichocladium</u> <u>asperum</u>	1
<u>Trichoderma</u> <u>alba</u>	25
<u>viride</u>	1994
<u>Trichothecium</u> <u>roseum</u>	3
<u>Triposporium</u> <u>myrtii</u>	1
<u>Tritirachium</u> <u>album</u>	1
<u>Ulocladium</u> <u>botrytis</u>	1
<u>atrum</u>	1
<u>tuberculatum</u>	3
<u>Verticillastrum</u> sp.	1
<u>Verticillium</u> spp.	31
<u>lateritium</u>	86
Unidentified <u>Dematiaceae</u>	425
<u>Moniliaceae</u>	4369
<u>Stilbellaceae</u>	8

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